CHARACTERIZATION OF THE ROLE OF THROMBOXANE IN THE AORTA AND KIDNEY AND THE THROMBOXANE RECEPTOR IN THE GLOMERULUS

BY

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Let no youth have any anxiety about the upshot of his education, whatever the line of it may be. If he keeps faithfully busy each hour of the working day, he may safely leave the final results to itself. He can, with perfect certainty, count on waking up some fine morning to find himself one of the competent ones of his generation, in whatever pursuit he may have singled out.

William James

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABBREVIATIONS

ACEI	angiotensin converting enzyme inhibitor
ACh	acetylcholine
ANOVA	analysis of variance
B_{max}	maximal binding
BP	blood pressure
cAMP	cyclic adenylate monophosphate
$[Ca^{2+}]_i$	intracellular free calcium concentration
DAG	diacylglycerol
EC_{50}	50% effective concentration
EDCF	endothelium-derived contracting factor
EDRF	endothelium-derived relaxing factor
FBF	femoral blood flow
FVR	femoral vascular resistance
GFR	glomerular filtration rate
GTP	guanylate triphosphate
Hct	hematocrit
HEPES	4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid
i.a.	intra-arterial
I-BOP	[1S-(1a,2b(5Z),3a(1E,3R*),4a)]-7- [3-(3-hydroxy-4-(4'-iodophenoxy)-1-butenyl)-7-oxabicyclo-[2.2.1]heptan-2yl]-5-heptenoic acid
IC_{50}	50% inhibitory concentration
i.p.	intraperitoneal
IP_3	inositol 1,4,5-triphosphate
I-PTA-OH	9,11-dimethylmethano-11,12-methano-16-(3-iodo-4-hydroxyphenyl)-13,14-dihydro-13-aza-15ab-w-tetranorthromboxane A_2
i.v.	intravenous
2K,1C	two kidney one clip
K_d	dissociation constant
$6kPGF_{1\alpha}$	6-keto-prostaglandin $F_{1\alpha}$
L-657,925 (- and +)	(-) and (+) -9-chlorobenzyl-6-fluoro-1,2,3,4-tetrahydrocarbazol-1-yl acetic acid
LY-163,443	1-[2-hydroxy-3-propyl-4-{[4-(1H-tetrazol-5-ylmethyl)-phenyl]methyl} ethane
MAP	mean arterial blood pressure
Meta-I-PTA-PO	9,11-dimethylmethano-11,12-methano-16-(m-iodophenoxy)-13,14-dihydro-13-aza-15ab-w-tetranorthromboxane A_2
NO	nitric oxide
NTG	nitroglycerin

ONO11120	9,11-dimethylmethano-11,12-methano-16-(phenyl)-13,14-dihydro-13-aza-15(a or b)-w-tetranorthromboxane A_2
PAH	para-aminohippuric acid
PBS	phosphate buffered saline
PG	prostaglandin
PGD_2	prostaglandin D_2
PGE_2	prostaglandin E
$PGF_{2\alpha}$	prostaglandin $F_{2\alpha}$
PGG_2	prostaglandin G_2
PGH_2	prostaglandin H_2
PGI_2	prostaglandin I_2
PLA_2	phospholipase A_2
PLC	phospholipase C
PRA	plasma renin activity
PTA-NO	9,11-dimethylmethano-11,12-methano-16-(2-naphthoxy)-13,14-dihydro-13-aza-15ab-w-tetranorthromboxane A_2
PTA-TPO	9,11-dimethylmethano-11,12-methano-16-thiophenoxy-13,14-dihydro-13-aza-15ab-w-tetranorthromboxane A_2
RGM	rat glomerular membranes
RBF	renal blood flow
RVH	renovascular hypertension
RVR	renal vascular resistance
SHR	spontaneously hypertensive rat
SQ-29,548	[1S-(1a,2b(5Z),3b,4a)]-7-{3-[[2-[(phenyl amino)carbonyl]hydrazino]methyl]-7-oxabicyclo[2.2.1]hept-2-yl}-5-heptenoic acid
TxA_2	thromboxane A_2
TxB_2	thromboxane B_2
U-46,619	15S-hydroxy-11a,9a(epoxymethano)prosta-5Z,13E-dienoic acid
UK-38,485	3-(1H-Imidazol-1-ylmethyl)-2-methyl-1H-indole-1-propanoic acid
UV	urine flow
WKY	Wistar-Kyoto rat
WRP	washed rat platelets

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These studies were planned to explore the mechanism of action of TxA_2 using whole animal, isolated tissue, and receptor binding techniques. The TxA_2 mimetic, U-46,619, caused a dose-dependent contraction of isolated aortic rings which was potentiated by endothelium removal or by hemoglobin. The release of TxB_2 from the rings was increased by U-46,619; inhibition of TxA_2 synthesis in vitro potentiated the response to U-46,619. These results indicated that contraction of rat aortic rings with TxA_2 was attenuated by EDRF and that endogenous vascular TxA_2 generation contributed to the contractile response.

Infusion of U-46,619 (1 $\mu g \cdot kg^{-1} \cdot min^{-1}$) in the anesthetized rat decreased glomerular filtration rate (GFR) and doubled renal vascular resistance (RVR), but femoral

vascular resistance was unchanged. These effects were prevented by a TxA_2/PGH_2 receptor antagonist. A TxA_2 synthetase inhibitor and α-adrenergic and leukotriene D_4/E_4 receptor antagonists blunted the renal hemodynamic responses. These results indicated that TxA_2 altered renal function by a receptor-mediated mechanism which is potentiated by TxA_2 release and by α-adrenoceptor and LTD_4/E_4 receptor activation.

Using the 2K,1C rat model of renovascular hypertension, renal TxB_2 excretion was increased during the early and the late phases, and hypertension was blunted by a TxA_2/PGH_2 receptor antagonist. These results indicate an important role of TxA_2 in this form of hypertension.

The TxA_2 mimetic [^{125}I]-BOP was used in binding studies to characterize the TxA_2/PGH_2 receptor in rat glomerular membranes (RGM) and rat mesangial cells. Equilibrium binding identified a single class of receptors of high affinity. The ED_{50} for phosphoinositide hydrolysis in rat whole glomeruli stimulated by I-BOP was comparable to the K_d from binding studies. Binding and phosphoinositide hydrolysis were antagonized stereoselectively by TxA_2/PGH_2 receptor antagonists. Six 13-azapinane TxA2 antagonists displaced [^{125}I]-BOP with a rank order of potency which was significantly different in RGM compared to washed platelets. These results indicated that I-BOP binds to a specific and functional receptor in RGM and that different subtypes of the TxA_2/PGH_2 receptor appear to exist in glomeruli and platelets.

CHAPTER 1
INTRODUCTION

The Role of Thromboxane A_2 and Prostaglandins in Renal Function

Prostaglandins (PGs) and TxA_2 are derived by the action of cyclooxygenase on arachidonic acid, which is a twenty-carbon, tetra-unsaturated fatty acid (Montgomery et al., 1983). Phospholipase A_2 (PLA_2) and C (PLC) cleave the sn-2 (sn, stereospecific numbering) position of cell membrane phospholipids to liberate arachidonic acid into the cytoplasm (Badr and Jacobson, 1991). Cyclooxygenase metabolizes arachidonic acid to the unstable intermediate endoperoxides PGG_2 and PGH_2. Specific enzymes--PGD_2 endoperoxide isomerase, PGE_2 endoperoxide isomerase, $PGF_{2\alpha}$ endoperoxide isomerase, prostacyclin synthetase, and TxA_2 synthetase--subsequently catalyze the formation of PGD_2, PGE_2, $PGF_{2\alpha}$, PGI_2, and TxA_2, respectively. These products readily diffuse across the cell membrane because of their high lipid solubility. The actions of the synthetizing enzymes regulate the rate of PG synthesis (Smith et al., 1983), since PGs are not stored within cells (Glew, 1982).

The PGs and TxA_2 are short-lived and function predominantly as local tissue hormones or autocoids. The net synthesis of different PGs within an individual cell, which

can determine its profile of biological responses, is dependent on the presense of cyclooxygenase and the endowment with the specific enzymes required for the synthesis of PGs and TxA_2, the activity of these enzymes, and the rate of degradation of PGs and TxA_2 in the cell. Thus the main product of cyclooxygenase in platelets is TxA_2, which functions as an aggregatory and vasoconstrictory agent, while a major PG product of the endothelium is PGI_2, which has the opposite profile of actions. The intermediate endoperoxide, PGH_2, shares many of the physiological actions of TxA_2 because it probably binds to the same receptor (Le Breton et al., 1979) (Figure 1-1). Prostaglandins activate adenylate cyclase to form the second messenger cyclic adenylate monophosphate (cAMP) and usually have anti-aggregatory and vasodilatory actions. In contrast, TxA_2 activates PLC to form two second messengers, inositol 1,4,5-triphosphate (IP_3) and diacylglycerol (DAG), to produce its aggregatory and vasoconstrictory actions. The IP_3 stimulates release of intracellular free calcium ($[Ca^{2+}]_i$) which can activate PLA_2 to catalyze conversion of phosphatidylcholine to lysophosphatidylcholine with release of arachidonic acid. The DAG can be converted to phosphatidic acid which may be hydrolyzed to lysophosphatic acid and arachidonic acid (Rasmussen, 1986). Therefore, TxA_2 can increase the turnover of arachidonic acid which can be converted to PGs and more TxA_2. It has been assumed that the balance in the production

of PGs and TxA_2/PGH_2 can modulate the physiological functions of blood vessels (Armstrong, 1983).

The biosynthesis of PGs and TxA_2 may be regulated at several steps. Phospholipase activity may be inhibited by glucocorticosteroids, whereas aspirin and indomethacin inhibit the formation of endoperoxides by cyclooxygenase. Our knowledge of the biological regulation of the specific enzymes catalysing the formation of the individual PGs and TxA_2 is poorly understood. However, TxA_2 synthetase may be inhibited by imidazoles, and TxA_2 receptor antagonists block the actions of PGH_2 and TxA_2 (Smith et al., 1983). The development of these specific drugs has been critical in probing the biological functions of cyclooxygenase products and of the TxA_2/PGH_2 receptor.

In the kidney, PGs and TxA_2 modulate renal blood flow (RBF) and GFR by actions on afferent arterioles, efferent arterioles, and the glomerulus. PGs modulate renal excretion of water and sodium by actions on transport function in the proximal tubule, loop of Henle, and the collecting tubule (Jackson et al., 1985; Welch and Wilcox, 1988a). Also PGs and TxA_2 may regulate RBF and GFR indirectly via actions on the tubuloglomerular feedback response (Welch and Wilcox, 1990) or by altering renin release (Welch et al., 1989). Inhibition of PG synthesis with indomethacin reduces RBF, GFR, renin secretion, naturesis, and diuresis (Lemley and Kriz, 1989). However, the actions of indomethacin depend on the physiologic state of the animal. The role of TxA_2 in

mediating changes in these vascular and tubular functions has not been completely established. Thus, PGs can modulate the effect of vasoconstrictors and, therefore, the actions of PGs depend on the profile of vasoconstrictor release. Cyclooxygenase inhibition has little effect on blood pressure (BP) or RBF in uninstrumented, conscious, salt-replete subjects. However, if angiotensin II release is increased by surgery, salt depletion, or anesthesia, indomethacin can raise BP and reduce RBF. While TxA_2 has only a modest action in regulating renal hemodynamics in normal, unstressed animals, elevated production of TxA_2 has been identified in certain disease states such as renovascular hypertension, chronic renal failure, and ureteral obstruction. In these diseases, TxA_2 exerts a pro-hypertensive or renal vasoconstrictive action. Indeed, TxA_2 can have decisive effects on BP and renal function (Lemley and Kriz, 1989).

Hormonal and Autocoid Control of Renal Hemodynamics

Renal glomeruli

Substantial PG and TxA_2 production in renal glomeruli has been demonstrated in numerous studies by measurement of the release of PGs or their metabolites from isolated glomeruli or glomerular cells in culture (Folkert and Schlondorff, 1979; Hassid et al., 1979; Sraer et al. 1979). The initial reports have concluded that three renal PGs are synthesized in rat glomeruli in the following order of

abundance: PGE_2 > $PGF_{2\alpha}$ > $6kPGF_{1\alpha}$ (Sraer et al., 1979). In a later study, conflicting results were reported which suggest that TxB_2 is the major prostanoid produced by rat glomeruli (Sraer et al., 1982). The cause for this variation in the measurements of TxB_2 has not been addressed, but in a more recent study, TxB_2 constituted 1.4% of total PG synthesis by rabbit glomeruli, while PGE_2 made up 60%, $PGF_{2\alpha}$ 22%, and PGI_2 17% (Farman et al., 1987). It is clearly shown in the latter study that the glomerulus produces more PGs and TxA_2 than other regions of the nephron, and that a larger proportion of the PGs and/or TxA_2 formed in the glomerulus is TxA_2. Therefore, while the precise quantification of TxA_2 generation in the glomerulus is variable, it is produced in the glomerulus and other portions of the nephron in significant quantities.

Receptor activation by PGs and TxA_2 has been identified in glomeruli by radiolabelled ligand binding techniques. Specific PGE_1 and PGE_2 receptors linked to adenylate cyclase are present in isolated rat glomeruli (Chaudhari and Kirschenbaum, 1985; Friedlander et al., 1983). Direct binding of PGI_2 within the glomerulus has not been demonstrated, but indirect evidence for PGI_2 receptors has been obtained by stimulation of cAMP production by PGI_2 (Friedlander et al., 1983). Specific TxA_2 binding has been characterized in rat glomeruli, although this binding phenomena has not been correlated with a functional response in this tissue (Wilkes et al., 1989). Therefore, not only

are there specific receptors for vasodilator PGs in glomeruli which are linked to a second messenger, but there are also binding sites for vasoconstrictor PGs. However, neither the function of these TxA_2 binding sites in the glomerulus nor the physiologic stimuli that elicit release of TxA_2 at this site have been clearly defined.

The functions of PGs and TxA_2 in the renal glomerulus and afferent arteriole and juxtaglomerular apparatus may involve alterations in glomerular filtration and renin secretion. Since the vasculature of the glomerulus is a major site of cyclooxygenase activity (Morrison, 1986), PG and TxA_2 production may exert effects on the vascular parameters of renal function. For example, vasoactive agents such as norepinephrine and angiotensin II release PGE_2 and PGI_2 from the glomerulus (Schlondorff, 1986). These vasodilatory PGs may offset constriction of blood vessels or mesangial cells stimulated by these agonists (Fujiwara et al., 1989). Indeed, PGE_2 applied to isolated glomeruli can decrease the reduction of glomerular planar surface area produced by angiotensin II, and TxA_2 and its analogues can decrease glomerular planar surface area which can be eliminated by a TxA_2 receptor antagonist (Scharschmidt et al., 1986). However, despite the finding that a TxA_2 analogue causes renal vasoconstriction and mesangial cell contraction (Mené and Dunn, 1986), it does not produce a consistent change in the glomerular capillary ultrafiltration

coefficient when the analogue is infused systemically (Baylis, 1987).

Some actions of PGs and TxA_2 on renal function are indirectly mediated by release of other agents. Thus, PGI_2 can stimulate renin release sufficiently to prevent renal vasodilation unless the actions of angiotensin II are blocked by an angiotensin II receptor antagonist (Scharschmidt and Dunn, 1983). In a different manner, TxA_2 released from the glomerulus can inhibit renin release (Welch et al., 1989). Moreover, TxA_2 can mediate a large fraction of the vasoconstrictor effects of infused angiotensin II (Wilcox and Welch, 1990a). Furthermore, endogenous TxA_2 modulates the tubuloglomerular feedback response (Welch and Wilcox, 1988a). Thus, release of PGs and TxA_2 from the glomerulus, renal arterioles, and tubules can be very important in mediating and modulating renal function. Further studies are required, however, to elucidate the details of these actions and the specific cells within the glomerulus which produce and respond to the various members of the family of PGs.

Mesangial cells

Mesangial cells are derived from smooth muscle and are located in the apices of the glomerular capillary tufts. Some mesangial cells have a phagocytic action which may be involved in clearing debris from the glomerular membrane filter. Others have a contractile function. The latter group can regulate the area of the glomerular capillary

filter, since the basement membrane which forms the filter is inserted directly into the mesangial cells. Thus, contraction of mesangial cells may limit the glomerular ultrafiltration coefficient and thereby reduce the nephron's GFR. Mesangial cells are a target for many vasoactive agents which alter GFR.

Prostaglandin and TxA_2 production in glomerular mesangial cells is characterized by a marked synthesis of PGE_2 and a significant synthesis of $PGF_{2\alpha}$, TxB_2, and $6kPGF_{1\alpha}$ (Sraer et al., 1982; Scharschmidt and Dunn, 1983; Ardaillou et al., 1985). A local action of PGs and TxA_2 in the mesangium is suggested by their generation in this tissue.

Specific receptors for PGs and TxA_2 have not been demonstrated in cultured glomerular mesangial cells by direct binding studies (FitzGerald et al., 1989). However, indirect evidence for $PGF_{2\alpha}$ and TxA_2 receptors has been derived from accumulation of inositol phosphates and an increase in $[Ca^{2+}]_i$ induced by $PGF_{2\alpha}$ and TxA_2. The increase in $[Ca^{2+}]_i$ after stimulation by a TxA_2 agonist can be inhibited by a TxA_2/PGH_2 receptor antagonist, although the effects of these antagonists on phosphoinositol hydrolysis have not been reported (Mené et al., 1988). Since no $PGF_{2\alpha}$ antagonists are available, it is unclear whether the actions of this PG are mediated via its own specific receptor.

Prostaglandins and TxA_2 can alter glomerular function by modulating contraction of the glomerular mesangial cells. Angiotensin II and arginine vasopressin reduce the single

nephron GFR and glomerular ultrafiltration coefficient. The striking increase in PGE_2 synthesis by mesangial cells following angiotensin II or arginine vasopressin stimulation may offset their effects on single nephron GFR and glomerular ultrafiltration coefficient (Scharschmidt et al., 1986). TxA_2 analogues and $PGF_{2\alpha}$ stimulate the contraction of mesangial cells, and TxA_2 constricts isolated glomeruli (Mené and Dunn, 1986; Dunn and Mené, 1989). Although an action of TxA_2 on the contractile properties of the mesangial cell and the glomerulus has been clearly demonstrated, a clear definition of the role of TxA_2 in glomerular hemodynamics and a clear description of its mechanism of action on the mesangial cell have not been provided.

Epithelial cells

Epithelial cells line both the glomerular membrane, where they are termed podocytes and Bowman's space where they are termed parietal epithelial cells. Podocytes have an important function in supporting the glomerular filter; podocytes are regularly deformed in kidneys from animals or patients with glomerular injury causing proteinuria. The function of the patietal epithelial cells is unclear. Moreover, which of the cell types constitutes the cells grown as primary cultures of glomerular epithelial cells is not clear. However, both types of glomerular epithelial cells are in direct continuity with the tubular fluid, and therefore any PGs synthesized by them are likely released

directly into the tubular lumen where they might modulate tubular cell function.

Prostaglandin and TxA_2 synthesis by renal epithelial cells has the same order of abundance as described for mesangial cells (Sraer et al., 1982). Angiotensin II stimulates PGE_2 release predominantly (Petrulis et al., 1981). However, no definitive role of PG or TxA_2 receptors has been provided for these cells nor is the function of PGs and TxA_2 in these cells understood.

Endothelial cells

Endothelial cells line the entire surface of the glomerular capillaries and are perforated by large fenestrae. These openings allow ready filtration of vascular fluid into Bowman's space while still retaining most of the blood cells in the vascular space. The endothelial cells are supported on a acellular basement membrane. As with other vascular endothelial cells, these cells in the glomerulus can no longer be considered passive barriers, but they are the first site of interaction of the components of the blood with the nephron. The glomerular endothelial cells may produce substances that can modify the filtered fluid further along the nephron.

The synthesis of PGs and TxA_2 in cultured glomerular endothelium and the presence of specific receptors in this tissue have not been reported. However, studies have shown the synthesis of PGE_2, $6kPGF_{1\alpha}$, $PGF_{2\alpha}$, and TxB_2 in cultured

bovine aortic endothelial cells (Ingerman-Wojenski et al., 1981). The synthesis of PGI_2 from cultured bovine aortic endothelial cells can be inhibited by the prostacyclin synthetase inhibitor, tranylcypromine (Weksler et al., 1977), while the TxA_2 release by cultured rabbit aortic endothelial cells can be inhibited by a calcium-channel antagonist, nifedipine (Ramadan et al., 1990). Recent studies have shown that endothelium-derived relaxing factor (EDRF) is released from bovine glomerular endothelial cells in culture by calcium-mobilizing agonists (Marsden et al., 1990). Since TxA_2 is also a calcium-mobilizing agonist in aortic endothelial cells, it may stimulate release of EDRF via a calcium channel on glomerular endothelial cells. TxA_2 also triggers release of PGI_2 from aortic endothelial cells (Hunt et al., 1989). Thus, TxA_2 may cause the coordinated release of both PGs and EDRF which act via discrete second messengers to prevent platelet aggregation and to diminish the vasoconstriction produced by angiotensin II, arginine vasopressin, and TxA_2 itself.

Renal resistance vessels

Rabbit renal microvessels synthesize PGI_2 at three times the rate of PGE_2 (Chaudhari and Kirschenbaum, 1988). PGs relax rabbit renal resistance vessels and offset the vasoconstrictor action of norepinephrine. Pretreatment with a cyclooxygenase inhibitor prevents endogenous PG production and abolishes the vasodilatory effects of arachidonic acid.

There is a segmental organization of the response to PGs within the renal microvascular tree; the interlobular arteries are most sensitive to PGE_2, the afferent arterioles are most sensitive to PGI_2, and the efferent arterioles respond only to PGI_2. Angiotensin II constricts the efferent arterioles, and this effect is attenuated only by PGI_2. PGD_2 has minor vasoconstrictor effects on interlobular arteries and afferent arterioles whereas $PGF_{2\alpha}$ has no effect on any segment of the renal resistance vessels (Edwards, 1985). Thus, vasodilator PGs are synthesized in renal resistance vessels where they exert important actions. Indirect evidence of PGI_2 and PGE_2 receptors has been presented since these PGs activate adenylate cyclase via a guanylate triphosphate-dependent process in rabbit preglomerular microvasculature. This observation implies that the vasodilatory effects of PGE_2 and PGI_2 are mediated through a receptor linked by a G-protein to adenylate cyclase (Chaudhari et al., 1990).

Synthesis of TxA_2 by renal resistance vessels has not been demonstrated. However, human coronary vessels release TxA_2 and PGI_2, and a TxA_2 synthetase inhibitor blocks the release of TxA_2 but not PGI_2 (Mehta and Roberts, 1983). Human blood vessels produce PGI_2 and TxA_2 in the ratio of 4:1 (Serneri et al., 1983). In the rabbit, PGI_2 is released from inner layers of the aorta whereas TxA_2 production is higher is the outer layers (Brunkwall et al., 1987). A TxA_2 analogue stimulates PGI_2 release from rat aorta by a

mechanism which is blunted by calcium channel antagonists (Jeremy et al., 1985). In isolated hydronephrotic rat kidneys, a TxA_2 agonist vasoconstricts renal afferent arterioles but not efferent arterioles; again, this vasoconstrictor action can be blocked by calcium channel antagonists (Epstein et al., 1988). Intravenous infusion of a TxA_2/PGH_2 receptor agonist reduces GFR and filtration fraction in isolated rat kidneys (Epstein et al., 1988). Therefore, TxA_2 has a preferential affect on preglomerular vessels by activation of voltage-dependent calcium channels. No direct evidence exists for PG or TxA_2 receptors in renal resistance vessels. However, TxA_2 receptors have been shown in rat aorta (Morinelli et al., 1990), cultured rat aortic smooth muscle (Masuda et al., 1991), and endothelial cells (Hanasaki et al., 1988) using established radio-ligand binding studies in vitro. Studies of cultured cells from renal resistance vessels provide further support for a functional role for PGs and TxA_2. Norepinephrine stimulates production of PGI_2 and PGE_2 by rabbit renal microvascular smooth muscle cells (Gupta et al., 1988), while arachidonic acid stimulates production of $PGE_2 > PGI_2 > PGI_2 > TxA_2$ from cultured rabbit renal microvascular endothelial cells (Chaudhari et al., 1989).

Renal tubules and interstitium

PGE_2 is the predominate PG in the tubules of the medulla. Its synthesis is greatest in the medullary

collecting duct while synthesis in the cortical collecting duct and descending limb of the loop of Henle is only about 25% as great. In rabbit microdissected tubules, greater than 90% of the prostanoid production in all tubular segments is PGE_2; nevertheless, $PGF_{2\alpha}$ and $6kPGF_{1\alpha}$ are synthesized at rates 20-fold lower than PGE_2, while TxA_2 production is 100-fold lower (Farman et al., 1987; Bonvalet et al., 1987). PGE_2 metabolism in the thick ascending limb of Henle's loop by prostaglandin E-9 ketoreductase may account for some of the $PGF_{2\alpha}$ in the urine (Miller et al., 1985). PGE_2 is the major PG generated in cultured rat renomedullary interstitial cells (Maridonneau-Parini et al., 1985). Thus, the vasodilatory PGs are the predominate prostanoids in both the tubular and interstitial cells of the renal medulla.

Receptors for PGE_2 have been identified by autoradiography in the outer medulla > the inner medulla > the cortex and can be reversibly down-regulated by a PGE_2 agonist (Limas and Limas, 1984; Limas and Limas, 1987). After treatment with indomethacin, PGE_2 receptor density is increased in the inner medulla and also in the cortex (Eriksen et al., 1987). In the medulla, the ascending limb of Henle has a greater density than the collecting duct, and no PGE_2 receptors are found in proximal tubules (Eriksson et al., 1990). Neither PGI_2 nor TxA_2 binding in tubules has been reported. However, the Madin Darby canine kidney cell line possesses specific binding sites for PGE_2 and $PGF_{2\alpha}$ which are cross-reactive. Two subtypes of PGE_2 receptor have been identified in the

canine kidney; the PGE_2 receptor in the outer medulla is associated with an inhibitory G-protein while in the papilla it is stimulatory for the guanylate triphosphate (GTP) effect. (Smith et al., 1987).

PGE_2 acts on tubules to promote naturesis and diuresis. PGE_2 released by the tubular epithelium probably binds to PGE_2 receptors on the cell in which it is synthesized or one in close vicinity. Arginine vasopressin and other vasoconstrictors stimulate the synthesis of PGs which may modify the effect of these substances on the tubule. The increase in water flux across cell membranes induced by arginine vasopressin is inhibited by PGE_2 suggesting that prostaglandins have a diuretic effect by antagonizing the effects of AVP in the medulla (Martinez and Reyes, 1984).

Role of Thromboxane A_2 Animal Modeds of Hypertension and Renal Disease

Several animal models of hypertension and renal disease have been used to study the role of TxA_2. Thus, derived models exhibit a change in TxA_2 after an intervention whereas some genetic models exhibit an abnormal function that can be related to TxA_2 metabolism. The derived models that have evidence of increased TxA_2 in the kidneys include the obstructed ureter model which has an increase in TxA_2 generation in the obstructed kidney, the reduced renal mass model of chronic renal failure which has an increased TxA_2 production in the remnant kidney, and the two kidney one clip

(2K,1C) model of renovascular hypertension which has an increase in TxA_2 in both the clipped and unclipped kidneys. In some of these models, inhibitors of TxA_2 synthesis or TxA_2/PGH_2 receptor antagonists improve renal hemodynamics and/or reduce BP (Purkerson et al., 1985; Himmelstein and Klotman, 1989).

The DOCA-salt model has an increased urinary excretion and renal cortical release of TxB_2, PGE_2, and $6kPGI_2$, but the functional importance of the finding is unclear since neither a TxA_2 synthetase inhibitor nor a TxA_2/PGH_2 receptor antagonist alter renal hemodynamics (Roman et al., 1988). The genetic models that have evidence of increased renal TxA_2 generation include the spontaneous hypertensive rat (SHR) and the Dahl-salt rat. In Dahl-salt sensitive rats, PGE_2 production is decreased in the collecting tubule, which may limit naturesis and contribute to volume-loading hypertension. Aortic TxA_2 is increased in the Dahl-salt sensitive rat; this finding, together with the underproduction of PGE_2 and the inability to regulate PGE_2 receptors, may contribute to the hypertension in this model.

Hypertension

The animal model of hypertension in which the role of prostaglandins and TxA_2 has been most extensively studied is the SHR derived from the Wistar-Kyoto rat (WKY) colony. Three areas of difference between SHR and its normotensive control may be related to the pathogenesis of this

genetically hypertensive model: 1) greater sympathetic nerve activity in SHR, 2) increased wall to lumen ratio in the blood vessels of SHR, and 3) a defect in ionic homeostasis in various cell types (Lovenberg, 1987). What particular derangement in cyclooxygenase product generation is manifested in SHR and which PGs and/or TxA_2 are involved has been controversial. However, recent studies have revealed several possible causes: enhanced phospholipase activation, post-receptor defects, defective modulation of noradrenergic neurotransmission, and defective synthesis of vasodilator prostaglandins.

Renal PLA_2 and PLC activity are enhanced in SHR causing an increase in PG production. This enhanced activity of the enzymes which release the substrate for PG production is increased with age providing increased arachidonic acid for PG synthesis in the kidneys of older SHR (Kawaguchi et al., 1987). PGE_2 is the major PG in both the cortex and the medulla of the kidney in SHR, while TxA_2 is found in the cortex. There is also a decrease in the PG to TxA_2 ratio which favors a predominance of vasoconstrictor effect (Kawaguchi et al., 1987). PG synthetase activity is increased in 10-12 week old SHR in the developmental stage (Dunn, 1976; Limas and Limas, 1977) and is increased further with age (Limas and Limas, 1977). PG metabolism by 15-hydroxyprostaglandin dehydrogenase is lower at all ages of SHR (Dunn, 1976; Limas and Limas, 1977; Nakanishi et al., 1986), and PG and TxA_2 synthesis is increased in glomeruli of

6-8 week and older SHR (Konieczkowski et al., 1983). Thus, the enzymes for synthesis and degradation of PGs and TxA_2 may play a role in the etiology of hypertension in the SHR.

The role of TxA_2 in the development of hypertension in the SHR is controversial. Treatment of young SHR in the pre-hypertensive stage with a TxA_2 receptor antagonist does prevent hypertension (Stegmeirer et al., 1987). In contrast, a TxA_2 synthetase inhibitor does not change GFR, renal plasma flow (RPF), or systolic BP in young SHR after acute or chronic administration (Grone et al., 1986). These results are in conflict with the conclusions of other studies which show that a continuous infusion of TxA_2 synthetase inhibitor is able to lower BP in 6 week old SHR to levels of control WKY rats (Stier and Itskovitz, 1988). Moreover, these drugs can lower systolic BP in the established phase of hypertension (Uderman et al., 1984). Similarly, a TxA_2 synthetase inhibitor can decrease the systolic BP and increase the GFR and RPF in SHR (Purkerson et al., 1986) and delay the initiation of hypertension by about one week (Shibouta et al., 1985).

The synthesis of PGs and TxA_2 in the SHR may be dependent on the stage of development of hypertension. The production of TxB_2 in aortic rings of 5 week old SHR is enhanced compared to WKY rats while the production of $6kPGF_{1\alpha}$ is not different between SHR and WKY rats. At 20 to 25 weeks of age, TxB_2 generation is not different between SHR and WKY rats, but $6kPGF_{1\alpha}$ synthesis is increased 2-fold (Osanai et

al., 1990). Thus, TxA_2 production in the young SHR may affect the development of hypertension, and PGI_2 production may moderate the hypertensive state in the older SHR.

Further studies suggest that there is an enhanced response to angiotensin II secondary to a deficiency of vasodilator prostaglandin in SHR and an enhanced response to TxA_2 due to increased vascular reactivity of SHR kidneys to endogenously released TxA_2. In addition, an increased TxA_2 release can cause a rapid proliferation of vascular smooth muscle cells in SHR which may be the structural component in maintenance of the hypertension (Ishimitsu, 1988b).

Several studies suggest that a defect in PGs is involved in the hypertensive state in SHR. While vasodilator PGs may actually rise with the development of hypertension, urinary excretion of PGE_2 were lower in SHR than in control WKY rats and the increase in the metabolite of PGI_2 in WKY rats on high salt diet is not present in SHR (Martineau et al., 1984). Other studies have shown that the PGE_2 receptor number is increased in the renal medulla of the 12 week old SHR which is consistent with up-regulation of the prostaglandin receptor in the presence of a deficiency of PGE_2. A post-receptor defect has been demonstrated by the following: (1) G-protein coupling of the PGE_2 receptor to adenylate cyclase is defective (Yoshikawa et al., 1990), (2) a defect in renal adenylate cyclase response to PGE_2 in kidneys of SHR has also been reported which may change the vasodilator and naturetic effects of PG release (Umemura et

al., 1985), and, additionally, (3) PGE_2 synthesis and cAMP were lower in cultured papillary collecting tubule cells of SHR than in WKY rats (Sato et al., 1989). These studies are consistent with a defect in the modulating effect of PGs during the developmental phase of hypertension in the SHR.

Recently a TxA_2-induced model of hypertension, has been investigated by Wilcox et al. (1990a) to clarify the role of TxA_2 in maintaining an elevated BP. Hypertension is produced by infusion of a TxA_2 mimetic which increases systolic BP in 10 days. Hypertension is abolished by anesthesia and by an α-adrenergic antagonist, which indicates a predominant role for central activation of the sympathetic nervous system (Ahlstrom et al., 1990). Stimulation of the sympathetic nervous system by the TxA_2 mimetics may account for a stimulus to the renin-angiotensin system which contibutes to the hypertension in this model. In another study, a central cardiovascular role of TxA_2 mimetic has also been shown by intracerebroventricular administration in SHR (Sirén et al., 1985). Thus, the role of TxA_2 in the etiology of hypertension is more complex than previously proposed.

Renovascular hypertension

Renovascular hypertension in human subjects is secondary to occlusive disease of the main renal arteries which may be caused by atherosclerosis or fibromuscular dysplasia. In the 2K,1C model of renovascular hypertension, the clipped kidney secretes renin, while the contralateral kidney undergoes

functional changes caused by the decreased excretory function of the clipped kidney and the increase in angiotensin secreted by the clipped kidney. The net effect is hypertension due to vasoconstriction and an inability of the kidneys to excrete sufficient sodium and water. The increased angiotensin may stimulate PG and TxA_2 release (Aiken et al., 1973). The released PG may attenuate the vasoconstriction, while TxA_2 may augment the vasoconstrictive actions of angiotensin. Indeed, the pressor response to angiotensin may be mediated in large part by TxA_2 (Wilcox et al., 1990a).

Other etiologies for renovascular hypertension may be present. (1) An alteration in the response to TxA_2 in the contralateral kidney has been shown by increased sensitivity to TxA_2 mimetic in the 1-2 week post-clip rat (Zimmerman et al., 1987). (2) The secretion of PG and TxA_2 by the clipped and unclipped kidneys is different. Isolated glomeruli of clipped kidneys produce greater amounts of PGs and TxA_2 than unclipped kidneys. Indomethacin decreases GFR in the clipped kidney but has no effect on GFR in the unclipped kidney (Stahl et al., 1984). This indicates a role for PG in maintaining renal function in the clipped kidney. (3) Another study showed that in the contralateral kidney there is an increase in renal TxA_2 production and a reduction in GFR. Since a TxA_2 synthetase inhibitor and a TxA_2 receptor antagonist can increase GFR in spite of a fall in systolic BP, this indicates that TxA_2 receptors are activated in the

contralateral kidney. Also functional hypertrophy of the contralateral kidney is prevented by a TxA_2 receptor antagonist which also alleviates the systemic hypertension (Himmelstein and Klotman, 1989). Clearly, the interaction of the renin angiotensin and PG/TxA_2 systems needs further investigation to clarify their mechanisms of action in renovascular hypertension and to improve the efficacy of therapeutic interventions.

Diabetes mellitus

There has been variability in the reports of PGs and TxA_2 production in streptozocin-induced diabetes. In one study of early diabetes, glomeruli had enhanced production of PGE_2, PGI_2, $PGF_{2\alpha}$, and TxA_2 (Craven et al., 1987; Schambelan et al., 1985). However, urinary excretion of TxA_2 is reported to be increased and PGE_2 and PGF decreased in another study (Bunke and Itskovitz, 1986). Similarly confusing are the reports that glomerular PGE_2 production is increased in streptozocin-induced diabetes but not in genetic diabetic rats (Barnett et al., 1987). Whether increased prostaglandin and TxA_2 synthesis has a significant role in the renal effects of diabetes is still controversial.

No direct ligand binding studies have been performed to investigate the receptor number or their sensitivity to PGs and TxA_2 in untreated or treated diabetes. TxA_2 receptor activation in diabetes mellitus has been suggested by the finding of an increased renal response to a TxA_2 agonist in

isolated perfused kidneys; this response is reduced by a TxA_2/PGH_2 receptor antagonist (Quilley and McGiff, 1990). Thus, increased TxA_2 generation and increased response to TxA_2 may occur in vascular and/or glomerular tissues in diabetes.

Prostaglandins in the kidney of diabetic animals may contribute to the glomerular hyperfiltration (Hostetter et al, 1982). In the early phase, there is an increase in GFR which is suppressed by treatment with indomethacin (Craven et al., 1987; Kirschenbaum and Chaudhari, 1986). However, while two studies report an increase in vasodilatory PGs, one study reports an increase in TxA_2 and the other a decrease in TxA_2. Therefore, the effect of diabetes on TxA_2 production, receptor number or tissue sensitivity in vascular and renal tissue and the linkage of receptor activation to the hemodynamic changes in diabetes remain to be resolved.

Objectives and Aims of this Research

The overall hypothesis that this work is designed to investigate is that TxA_2 regulates renal hemodynamics and function via actions on discrete receptors linked to second messengers dictating cell functions and that this train of events is perturbed in certain models of hypertension. This hypothesiss will be approached by a set of four objectives. (1) We will investigate the role of the vascular endothelium in the response to TxA_2. The specific aims are as follows.

We will determine the effects of the endothelium in governing the sensitivity of aortic vascular smooth muscle to TxA_2. Is the sensitivity to a TxA_2 mimetic dependent on the presence of an intact endothelium? Can any effects of the endothelium in modulating the sensitivity to TxA_2 be ascribed to release of nitric oxide (NO)?

We will determine the role of local tissue PG and TxA_2 production from aortic rings in modulating the vasoconstrictive response to TxA_2. Does a TxA_2 mimetic stimulate arachidonic acid release, with the formation of PGs and TxA_2 in the blood vessel wall, as it does in the platelet? Do products of cyclooxygenase or TxA_2 synthetase released in response to a TxA_2 mimetic modulate the vascular response to that mimetic?

(2) We will investigate the actions of TxA_2 on renal hemodynamics. The specific aims are as follows.

We will determine the role of TxA_2 in modulating renal function. Is the effect of an infusion of a TxA_2 mimetic on renal function mediated via a specific TxA_2/PGH_2 receptor? Are there parallel effects on GFR and RBF? What are the effects of an infusion of a TxA_2 mimetic on tubular function?

We will determine the role of endogenous renal PG and TxA_2 generation in modulating the renal response to an infusion of a TxA_2 mimetic. Does an infusion of a TxA_2 mimetic stimulate the release of renal PG and TxA_2

within the kidney? Which are the predominate PGs released? Are the net effects of cyclooxygenase products released by a TxA_2 mimetic vasoconstrictive in the rat kidney? Does enhanced renal TxA_2 generation contribute to TxA_2 mimetic-induced renal vasoconstriction?

We will determine the roles of other, non-PG, arachidonic acid metabolites in modulating the renal vasoconstrictor response to TxA_2. Can the effects of an infusion of a TxA_2 mimetic be altered by blocking lipoxygenase?

We will determine the role of sympathetic nervous system activation in mediating the renal vasoconstrictor response to TxA_2. Can the effects of an infusion of a TxA_2 mimetic be blocked by an α-adrenergic receptor antagonist?

We will determine the effects of TxA_2 on the systemic and renal vasculature. Is the responsiveness of vascular tissue to a TxA_2 mimetic different in renal blood vessels compared to systemic blood vessels? Are the differences between systemic and renal vascular responses to a TxA_2 mimetic due to organ-specific modulation by products of cyclooxygenase, TxA_2 synthetase, lipoxygenase or the sympathetic nervous system?

(3) We will investigate the role of TxA_2 in the 2K,1C model of renovascular hypertension. The specific aims are as follows.

We will determine the role of TxA_2 in the clipped compared to the unclipped kidney. Is overproduction of TxA_2 present in both ipsilateral and contralateral kidneys? Does overproduction of TxA_2 alter renal clearances in either kidney?

We will determine the role of TxA_2 in renovascular hypertension. Is TxA_2 elevated throughout the development of hypertension in this model? Are TxA_2 antagonists and TxA_2 synthetase inhibitors effective in ablating the hypertensive state? Is renovascular hypertension responsive to TxA_2 inhibition in the early and late stages of renovascular hypertension?

We will determine the interaction of TxA_2 and angiotensin II in renovascular hypertension. Is overproduction of TxA_2 secondary to angiotensin generation? Are angiotensin converting enzyme inhibitors effective during particular stages of renovascular hypertension?

(4) We will investigate the presence and function of TxA_2 receptors in the rat glomerulus. The specific aims are as follows.

We will determine the characteristics of specific binding of TxA_2 in the glomerulus. Do glomerular membranes exhibit saturable and high-affinity binding

with a TxA_2 mimetic? Is this binding displaceable by TxA_2/PGH_2 receptor agonists and antagonists? Does this binding display stereoselectivity?

We will determine the effects of TxA_2 on a biochemical response in the same tissue as used for binding studies. Is there an increase in phosphoinositide hydrolysis with TxA_2 mimetics in rat glomeruli? Is this biochemical response dose-dependent and of high efficacy? Is the ED_{50} for an agonist comparable to the dissociation constant (K_d) from binding studies? Can the response be competitively inhibited by TxA_2 receptor antagonists? Is there a correlation between drug efficacy in displacing ligand in binding studies and in inducing phosphoinositide hydrolysis in functional studies? Is stereoselectivity of binding reflected in stereoselectivity in the functional response?

We will determine if distinct TxA_2/PGH_2 receptor subtypes exist in the rat glomerulus compared to other tissues. Are the IC_{50} values of a homologous series of antagonists different in rat glomeruli compared to rat platelets or human platelets? Is the rank order of potencies for binding in glomeruli different than in platelets and vascular smooth muscle?

We will determine which specific cell type, among those that constitute the glomerulus, expresses the TxA_2/PGH_2 receptor. Is there specific binding of a TxA_2

ligand, with dose-dependent displacement by competing antagonists, in glomerular mesangial and/or epithelial cells in culture? Is the same biochemical response of phosphoinositide hydrolysis in response to TxA_2 mimetics also identified in mesangial cells as in the glomerulus, and is it inhibited with drugs which are competitive antagonists of the binding of the TxA_2 ligand in whole glomeruli?

These specific aims require investigation using a hierarchy of pharmacologic techniques, including: (a) isolated vascular tissue in an organ bath, (b) intact kidneys with infusion of agonists and antagonists, (c) whole animal models of renovascular hypertension, (d) glomerular cell membranes for receptor binding with radioligands, and (e) isolated glomeruli for measuring phosphoinositol hydrolysis. These aims have been designed to achieve the long-term goal which is to characterize the role of TxA_2 in the kidney and blood vessels of both healthy and diseased animals.

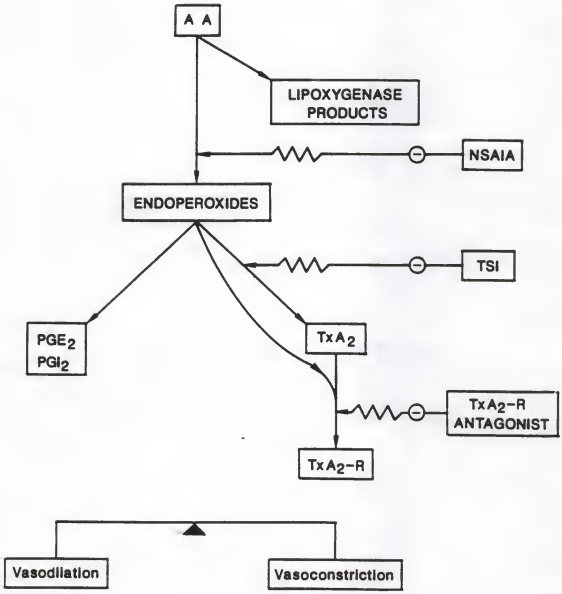

Figure 1-1. Cascade of arachidonic acid metabolism to PGs and TxA_2: balance between vasodilation and vasoconstriction.

AA, arachidonic acid; NSAIA, non-steroidal anti-inflammatory analgesics; TSI, TxA_2 synthetase inhibitor; TxA_2-R, Thromboxane A_2 receptor.

CHAPTER 2
RESPONSE OF RAT THORACIC AORTIC RINGS TO THE THROMBOXANE A_2 MIMETIC U-46,619: ROLES OF EDRF AND THROMBOXANE A_2 RELEASE

Introduction

TxA_2 is both a platelet-aggregating and a vasoconstrictor agent (Moncada and Vane, 1979a). Arachidonic acid can be metabolized via the cyclooxygenase and TxA_2 synthetase enzymes to TxA_2 in large and small vessels (Mehta and Roberts, 1983; Ishimitsu et al., 1988a). TxA_2 is produced mainly by the media of intact blood vessels (Brunkwall et al., 1987), although some is synthesized by cultured vascular endothelial cells (Ingerman-Wojenski et al., 1981). Recent evidence suggests an important role for TxA_2 in physiologic and pathologic control of vascular tone. For example, angiotensin II increases the production of TxA_2 which contributes to the ensuing renal and systemic vasoconstriction (Welch and Wilcox, 1988b; Wilcox and Welch, 1990b). Moreover, there is evidence of increased TxA_2 production in the kidneys or blood vessels from rats with various forms of hypertension, such as the spontaneously hypertensive rats (SHR) (Ishimitsu et al., 1988a, 1988b), renal parenchymal hypertension (Purkerson et al., 1985), Dahl salt-sensitive hypertension (Yamashita et al., 1988), and renovascular hypertension (Stahl et al., 1984). In some of

these models, inhibition of TxA_2 synthetase or receptors can ameliorate hypertension and/or renal vasoconstriction (Stahl et al., 1984; Purkerson, et al., 1985; Ishimitsu et al., 1988a, 1988b; Yamashita et al., 1988). Recent evidence suggests that endothelium of vessels from hypertensive rats releases endothelium-derived contracting factors (EDCFs) such as endothelin, PGH_2, or superoxide radicals. Pretreatment of vascular muscle with inhibitors of cyclooxygenase can blunt the release of EDCF (Miller and Vanhoutte, 1985). While endothelium-dependent contractions of the aortic muscle of the SHR are not prevented by inhibition of TxA_2 synthetase, they are blunted by a TxA_2/PGH_2 receptor antagonist (Auch-Schwelk et al., 1990). Therefore, a cyclooxygenase-dependent contracting factor, which appears to be distinct from TxA_2 but activates a similar receptor, may be released by endothelium of vessels from hypertensive rats.

TxA_2 increases $[Ca^{2+}]_i$ and activates phospholipase C in platelets (Pollock et al., 1984), vascular endothelial cells (Hunt et al., 1989), and renal mesangial cells (Mené et al., 1988). This may lead to activation of phospholipase A_2 and release of arachidonic acid with subsequent generation of PGI_2 and TxA_2 (Mehta et al., 1984; Jeremy et al., 1985; Hunt et al., 1989). Endothelial PGI_2 release has been implicated in moderating the contractile action of U-46,619 on dog coronary arteries (Szwajkun et al., 1990). The role of endogenous TxA_2 release in modulating the contractile response of smooth muscle to a TxA_2/PGH_2 mimetic is

investigated in the present series. Moreover, recent studies suggest that endothelium may also modulate the contractile response to a variety of agonists by release of EDRF (Dinerman and Mehta, 1990). Indeed, the vascular responses to platelets are modulated by the presence of the endothelium (Cohen et al., 1983; Houston et al., 1986) and endothelial removal (Szwajkun et al., 1990); atherosclerotic injury to the endothelium potentiates the response to U-46,619 (Lopez et al., 1989). Therefore, a second aim of these investigations is to study the role of the endothelium and EDRF in the contractile response of thoracic aortic rings to a TxA_2/PGH_2 mimetic.

Methods

Preparation

Male Sprague-Dawley rats (150-200 g) were sacrificed by inhalation of halothane in a bell jar. The thoracic aorta was harvested through a left thoracic incision and placed in a bath of Krebs' physiologic solution gassed with 95% O_2 and 5% CO_2 and maintained at 37°C. After removal of loose connective tissue, the aorta was cut into 4 segments of about 4 mm in length. Each was suspended in a custom-designed 2 ml chamber and connected to a force transducer (Kistler-Morse, Redman, WA). Each chamber was filled with gassed, physiologic Krebs' solution and maintained at 37°C. The

endothelium was removed from some aortic segments by gently rolling one tip of a microdissecting forceps placed inside the vessel. The paired segment retained its endothelium intact. The segments were subjected to 5 g of preload. The preload on the rings was maintained at 5 g because in multiple previous studies in rat aortic rings (n=150), this preload provided the optimum dose-response relationship to a variety of agonists. The presence (or absence) of intact endothelium was assessed by the characteristic relaxation (or absence thereof) in response to acetylcholine (ACh) (Furchgott and Zawadzki, 1980).

Protocol

The aortic rings stabilized for 1 hour while the Krebs' buffer in the organ bath was changed every 15 min. An aliquot (200 µl) was taken from the last supernatant and replaced with an equal volume of Krebs' solution. This aliquot was frozen at 40°C prior to TxB_2 assay. Thereafter, the TxA_2/PGH_2 mimetic U-46,619 (20 µl dissolved in Krebs' physiological solution) was added to the organ bath. The initial dose was 10^{-12} M; after a steady response was achieved, further doses of U-46,619 were added to give concentrations of 10^{-11}, 10^{-10}, 10^{9}, 10^{-8}, 10^{-7} M. An aliquot of the cumulative dose of U-46,619 at 10^{-7} M was frozen for subsequent assay. Some rings were tested with a second dose of U-46,619 at 10^{-8} M. Twenty µl of ACh (10^{-6} M) was added in

the presence of the highest dose of U-46,619 to check the integrity of the endothelium. Thereafter, 20 µl solutions of nitroglycerin (NTG; 10^{-8}, 10^{-7}, and 10^{-6} M) were added to the organ bath to verify the viability of the vascular smooth muscle. NTG produced smooth muscle relaxation in all rings studied.

Experimental Groups

Series 1

The aim of this series was to assess the role of the endothelium and EDRF in the contractile response to activation of TxA_2/PGH_2 receptors. Aortic rings were taken from rats without any pre-treatment. One half (n=14) were studied with the endothelium intact; one ring failed to relax with ACh and subsequent data with this ring were discarded. The other half (n=14) were studied with the endothelium denuded. The effects of oxyhemoglobin (10^{-6} M) added to the organ bath were studied in four rings (Palmer et al., 1987); their responses to U-46,619 were assessed as described above. In four experiments, SQ-29,548, which is a competitive antagonist of TxA_2/PGH_2 receptors (Ogletree et al., 1985), was added to the bath in a concentration of 10^{-6} M, and its effects on the contractile responses to U-46,619 was studied.

Series 2

The aim of this series was to assess the effect of inhibition of TxA_2 synthesis *ex vivo* on the contractile

response of aortic rings to U-46,619. To the supernatants of the bathing aortic rings were added either a TxA_2 synthetase inhibitor, UK-38,485, (Cross et al., 1986) at a concentration of 10^{-6} M or its vehicle. As in the previous series, one half of the rings had the endothelium removed. These studies followed the protocol described in series 1.

Series 3

The aim of this series was to assess the effects of a more prolonged in vivo inhibition of TxA_2 synthesis on the contractile response of vascular smooth muscle to U-46,619. Rats received UK-38,485, 50 $mg.kg^{-1}.d^{-1}$ intraperitoneally (i.p.) for three days before harvesting the aorta. The last dose was given on the morning of sacrifice. We showed previously that this dose of UK-38,485 reduces TxB_2 excretion by more than 70% without altering the excretion of $6kPGF_{1\alpha}$ (Welch and Wilcox, 1988b). The vehicle for UK-38,485 was administered to four other rats which formed the control groups for this study. As in the previous series, the endothelium was removed from half of the rings prior to study. Data for the three different series were analyzed seperately because experiments in these series were undertaken some weeks apart and the quantitative responses of the aortic rings to U-46,619 varied somewhat between the series.

Drugs Used

U-46,619 (Upjohn, Kalamazoo, MI) was dissolved in Krebs' physiological solution. UK-38,485 (Pfizer Central Research, Groton, CT) was dissolved in 1 N NaOH at pH 12.5 and titrated with 1N HCl to pH 8.5 at 100 $mg \cdot ml^{-1}$. SQ-29,548 (Squibb Institute for Medical Research, Princeton, NJ) was dissolved in 95% ethanol and 100 mM Na_2CO_3 and diluted with 0.15 M NaCl.

PGI_2 and TxA_2 Assay

The aliquots of supernatant from aortic rings with the endothelium were thawed, acidified to pH 3 with HCl, and extracted into 8 volumes of ethyl acetate on the day of the assay. The organic phase was evaporated to dryness with N_2, resuspended in buffer, and assayed using a specific radioimmunoassay. The assay characteristics and the details of the method used have been described in detail (Welch and Wilcox, 1988a).

Conversion of [^{14}C]-arachidonate to TxA_2 and PGI_2

Aortic rings with intact endothelium were prepared from rats and incubated in 3 ml of aerated Krebs' solution as in the experiments described above. Two sets of rings were prepared simultaneously. U-46,619 (10^{-6} M) was added to one bath while the other received a vehicle. [^{14}C]-arachidonate was obtained from New England Nuclear Research Products

(Boston, MA; specific activity 51.3 $mCi \cdot mmol^{-1}$) and 30 µl was added to each set of rings. An aliquot of both bath fluids was obtained for radioactivity counting before incubation. After allowing 120 min for incorporation at 37°C, aliquots of bath fluids were obtained for total activity counting and HPLC separation of the radioactive products.

Aliquots (1 ml) of bath fluid were spiked with $[^3H]$-$6kPGF_{1\alpha}$ and $[^3H]$-TxB_2 to assess recovery. They were acidified to pH 3-4 with 0.4 N HCl and extracted with ethylacetate. After drying under N_2, the residue was dissolved in 100 µl of 100% methanol. One third of the volume was injected into an HPLC (LDC/Milton Roy, Tampa, FL). The prostaglandins were separated according to the procedure of Henke et al. (1984). The mobile phase consisted of a series of isocratic elutions with mixtures of methanol, water, and acetic acid at a flow rate of 3 $ml \cdot min^{-1}$. Injections of $[^3H]$-TxB_2 and $[^3H]$-$6kPGF_{1\alpha}$ were run before each sample to verify retention times. Sample chromatographs were counted and plotted using a radiochromatographic detector (Flo-one Beta, Radiomatic Instruments Inc., Tampa, FL).

The percent conversion of arachidonate to TxB_2 and $6kPGF_{1\alpha}$ was assessed by factoring the radioactive counts in the peaks of $[^{14}C]$-$6PGF_{1\alpha}$ and $[^{14}C]$-TxB_2, corrected for recovery, by the total radioactive uptake of the tissue.

Statistics

Mean ± SEM values from multiple measurements are presented. For each ring, the concentration of U-46,619 required to increase the tension to 50% of maximum (ED_{50}) was obtained from individual dose-response curves. Between-group analyses were compared by paired t-tests for release of TxB_2. Data for ED_{50} responses were compared by Wilcoxon signed-rank or Mann-Whitney U tests, since the variances in some of the comparisons were different. Values of $p<0.05$ were taken as statistically significant.

Results

For each series, a sigmoidal relationship between increased force and dose of U-46,619 was observed.

Series 1

As shown in Figure 2-1 and Table 2-1, the contractile response to U-46,619 of rings whose endothelium was removed was accentuated by more than 1 log-fold compared to those with endothelium (ED_{50}: $4.78 \pm 2.14 \times 10^{-10}$ M versus $6.54 \pm 3.02 \times 10^{-9}$ M; $p<0.05$). After maximal contraction, the rings with endothelium relaxed in response to ACh while those without endothelium did not. However, all rings relaxed in response to NTG (data not shown).

The effects of addition of oxyhemoglobin to the organ bath on the response to U-46,619 was assessed in four aortic

rings. The ED_{50} of U-46,619 for the rings with endothelium was 6.63 ± 0.38 x 10^{-11} M which was the same as the ED_{50} of 5.13 ± 0.18 x 10^{-11} M for those without endothelium. After oxyhemoglobin, neither sets of rings relaxed with ACh.

SQ-29,548 (10^{-6} M) was added to two rings with and two without endothelium. After SQ-29,548 there was no contraction with U-46,619 in any ring.

Addition of U-46,619 to the organ bath containing aortic rings with endothelium increased the release of the TxA_2 metabolite, TxB_2, from 147 ± 12 to 211 ± 26 pg·ml^{-1} (n=13; $p<0.05$) and of the PGI_2 metabolite, $6kPGF_{1\alpha}$, from 293 ± 49 to 664 ± 91 pg·ml^{-1} (n=14; $p<0.01$) (Table 2-2). In this group, the release of TxB_2 into the supernatant was 175 ± 22 pg·ml^{-1} before addition of SQ-29,548 and was 155 ± 36 pg·ml^{-1} (n = 4, n.s.) after addition of SQ-29,548 to the bath.

The addition of U-46,619 (10^{-6} M) to the organ bath increased the fraction of [^{14}C]-arachidonate converted to [^{14}C]-TxB_2 from 0.14% to 0.74%, while the fraction converted to [^{14}C]-$6kPGF_1$ increased from 1.55 to 2.34%.

Series 2

The data for this series are shown in Figure 2 and Table 2-1. The responses of the rings which had vehicle added to the bath were qualitatively similar to those in series 1. Addition of UK-38,485 to the bath reduced the sensitivity of the rings with and without endothelium to U-46,619 without impairing the maximal response. As shown in Table 2-1, ED_{50}

values of the rings with and without endothelium were increased significantly by three- and fivefold, respectively, but there remained a significant difference between the rings with and without endothelium.

The TxB_2 concentration of the bathing fluid was measured in four preparations treated with vehicle and four with UK-38,485 (Table 2-2). The concentration in the fluid that had received vehicle increased after U-46,619 (59 ± 8 to 84 ± 13 $pg \cdot ml^{-1}$; $p<0.05$), while in the fluid bathing rings which received UK-38,485 TxB_2 was not increased following U-46,619 (66 ± 7 to 75 ± 11 $pg \cdot ml^{-1}$; n.s.).

Figure 2-3 shows examples of an individual experiment from series 2 (panel A) and series 3 (panel B). Panels A and B both show that in vehicle-treated rings or animals (solid lines), rings that had the endothelium removed began to contract at a rather lower dose of U-46,619, yet achieved a similar maximal response compared to rings with endothelium. Panel A also shows that after the addition of UK-38,485 to the bath (broken lines), the dose of U-46,619 required to initiate contraction was increased in both rings without affecting the maximal response. Panel B shows that a ring with endothelium taken from an animal pretreated with UK-38,485 for 3 days had a similar response to one without endothelium from a vehicle pretreated animal. However, a ring without endothelium from a UK-38,485 pretreated animal required a greater dose of U-46,619 to initiate contraction and had a reduced maximal response compared to its control.

Series 3

The group data for series 3 are displayed in Figure 2-4 and Table 2-1. In vehicle-pretreated rats, the responses of rings to U-46,619 were qualitatively similar to those in series 1. A three day pretreatment with the TxA_2 synthetase inhibitor reduced the sensitivity ($p<0.05$) and maximal response ($p<0.02$) of rings without endothelium to U-46,619 without significantly changing the response of those with endothelium. The ED_{50} values of rings without endothelium taken from animals that had received three days of pretreatment with UK-38,485 was increased by 5-fold (vehicle, $0.34 \pm 0.02 \times 10^{-9}$ M; UK-38,485, $1.65 \pm 0.48 \times 10^{-9}$ M; $p<0.05$), as shown in Table 2-1. In contrast, the ED_{50} of rings with endothelium taken from rats given the TxA_2 synthetase inhibitor was not significantly different from those that had received the vehicle ($2.18 \pm 0.51 \times 10^{-9}$ M versus $2.93 \pm 0.16 \times 10^{-9}$ M; n.s.). However, after pretreatment with the TxA_2 synthetase inhibitor, the rings without endothelium remained consistently more sensitive to U-46,619 than rings with endothelium ($p<0.05$).

The effects of the TxA_2 synthetase inhibitor on the release of TxA_2 and PGI_2 into the supernatant during U-46,619-induced contraction are shown in Table 2-2. Three days of pretreatment with the TxA_2 synthetase inhibitor did not significantly alter the concentration of TxB_2 in the supernatant before U-46,619 (vehicle-treated rats: 449 ± 252

versus UK-38,485-treated rats: 32 ± 21 $pg \cdot ml^{-1}$; n.s.) but significantly reduced the concentration after U-46,619 (vehicle-treated rats: 556 ± 239 versus UK-38,485 treated rats: 11 ± 9 $pg \cdot ml^{-1}$; $p<0.05$).

Discussion

The results of this study demonstrate that the TxA_2/PGH_2 mimetic U-46,619 produces a dose-dependent contraction of rat aortic smooth muscle at low concentrations and that this response is abolished by the selective TxA_2/PGH_2 receptor antagonist, SQ-29,548. This observation suggests that U-46,619 binds to specific, high-affinity, receptors in the aorta (Ogletree et al., 1985). Receptors have been identified using [^{3}H]-SQ-29,548 binding in human umbilical vessels (Hedberg et al., 1989) and rat glomerular membranes (Wilkes et al., 1989); U-46,619 displaces the bound ligand in a competitive manner.

In the present study, the smooth muscle contractile response to U-46,619 after endothelium removal is a 5- to 13-fold increase in sensitivity. This elevation in sensitivity cannot be ascribed to trauma since the relaxation response to NTG is unaffected. ACh causes relaxation of the aortic rings with endothelium which are contracted with U-46,619, implying that the endothelium is functionally intact and capable of releasing EDRF (Furchgott and Zawadzki, 1980; Palmer et al., 1987).

NO can account for many of the biological actions of EDRF (Palmer et al., 1987). NO is a powerful vasorelaxant whose effects are antagonized by oxyhemoglobin (Palmer et al., 1987). In keeping with this concept, treatment of aortic rings with oxyhemoglobin enhanced the vascular smooth muscle response to U-46,619, prevented ACh-induced vasorelaxation of rings with endothelium, and abolished the difference in response to U-46,619 between rings with and without endothelium. This finding indicates, albeit indirectly, that a major mechanism of the attenuated contractile response to U-46,619 of rings with endothelium may be due to basal endothelial NO release or to release stimulated in response to the TxA_2 mimetic. Oxyhemoglobin does not prevent the vasorelaxant actions of PGI_2 released from the endothelium (Palmer et al., 1987). Oxyhemoglobin greatly potentiates the sensitivity of aortic rings to U-46,619 and abolishes the differences in response between rings with or without endothelium. These observations imply that NO, rather than PGI_2, is functionally the most important EDRF in these studies. Unlike classical releasers of EDRF, such as ACh (Furchgott and Zawadzki, 1980), the effect of NO released with U-46,619 is to moderate the vasoconstriction rather than to produce a net vasorelaxation. This effect is analogous to the blunting of the contractile effects of norepinephrine and serotonin in coronary arteries (Cocks and Angus, 1983) and rat aortic rings (Dinerman and Mehta, 1990) by EDRF.

Blood vessels are significant sites of production of prostaglandins (Moncada and Vane, 1979; Ingerman-Wojenski et al., 1981; Mehta et al., 1984; Jeremy et al., 1985). Results from the present studies confirm that U-46,619 stimulates the release of PGI_2 and TxA_2 from rat aortic tissue. U-46,619 increases the release of immunoreactive metabolites of PGI_2 and TxA_2 into the supernatant of vascular rings exposed to U-46,619, and these results were confirmed by one experiment in which it increased the conversion of [^{14}C]-arachidonate to [^{14}C]-6kPGF$_{1\alpha}$ and [^{14}C]-TxB_2.

The role of PGs and vascular endothelium in the response to U-46,619 has been studied recently in vivo in dog coronary arteries by Szwajkun et al. (1990). Our results confirm their finding that the contractile responses to U-46,619 are moderated by the presence of the endothelium. Those authors found that indomethacin consistently augmented the response of the dog coronary artery to U-46,619, although it failed to prevent the full effects of endothelial removal. The authors provided no direct evidence for EDRF or prostaglandin release, nor did they use specific inhibitors of NO, but they concluded that vessel wall-derived prostaglandins and EDRF together modulated the coronary vascular response to U-46,619 in vivo. Our finding that oxyhemoglobin abolished the differences in contractile responses to U-46,619 between rings with and without endothelium confirm the conclusion of Szwajkun et al. (1990) of the importance of EDRF in moderating the vascular response to U-46,619. Our data also

clearly showed that U-46,619 releases PGI_2 and TxA_2 from rat thoracic aortic rings in vitro. Since the importance of U-46,619-induced TxA_2 release had not been assessed previously, further studies were undertaken to evaluate its role using a specific TxA_2 synthetase inhibitor.

Both the addition of a TxA_2 synthetase inhibitor ex vivo to the supernatant of aortic rings and three days of pretreatment of rats with this drug reduced the response of rings without endothelium to U-46,619 by more than 4-fold (Figures 2-2 and 2-4). This reduction indicates that U-46,619 stimulates the production or release of a factor by vascular smooth muscle that augments the contractile response and that this factor is blocked by inhibition of TxA_2 synthetase. This factor is most likely TxA_2, since UK-38,485 reduces TxB_2 release profoundly in vivo. However, the dose-response relationship between TxA_2 release and concentration of U-46,619 was not established in these studies. The decreased response to U-46,619 after pretreatment with the TxA_2 synthetase inhibitor cannot readily be explained by redirection of PGH_2 into PGI_2 synthesis, since UK-38,485 pretreatment did not cause a measurable change in $6kPGF_{1\alpha}$ release from the aortic rings. Moreover, PGI_2 is produced predominantly by vascular endothelium (Moncada and Vane, 1979a) while the effects of the TxA_2 synthetase inhibitor given for three days in series 3 are confined to rings without endothelium. In contrast, TxA_2 is produced predominantly by smooth muscle (Brunkwall et al., 1987),

although endothelial cells in culture can produce both PGI_2 and TxA_2 (Hunt et al., 1989). In view of this finding, we have assessed the effects of TxA_2 release on renal vasoconstriction induced by infusion of U-46,619 into intact rats. In this model, also, U-46,619 increases TxA_2 release (as assessed from TxB_2 excretion in the urine) and that endogenous TxA_2 contributes to the renal vasoconstriction induced by U-46,619 (see Chapter 3).

While the sensitivity of rings with endothelium to U-46,619 was not altered by three days of administration of the TxA_2 synthetase inhibitor in vivo, it was reduced when the inhibitor was delivered via the bathing fluid ex vivo. The addition of UK-38,485 to the bath blocked fully the increase in TxB_2 release induced by addition of U-46,619, while 3 days of UK-38,485 produced a profound depression of TxB_2 release from the rings both before and after addition of U-46,619. Exposure of platelets to U-46,619 leads to a rapid down-regulation of TxA_2/PGH_2 receptors and a decrease in agonist-induced calcium release (Murray and Fitzgerald, 1989). Therefore, it is possible that three days of pretreatment of rats with a TxA_2 synthetase inhibitor reduces endogenous TxA_2 sufficiently to up-regulate TxA_2 vascular receptors. This may offset the blunting of the response to U-46,619 which is seen after UK-38,485 is added directly to the tissue supernatant. However, this difference between the effects of in vivo and ex vivo administration of the TxA_2 synthetase inhibitor on the response to U-46,619 was not seen in rings

without endothelium, and the reason for it remains unexplained.

The endothelium also produces a number of contracting factors, including endothelin, angiotensin II, histamine, and TxA_2 (Lüscher, 1989). There is also evidence for two other less well defined EDCFs: $EDCF_1$ is a nonprostanoid substance which is released during hypoxia (Lüscher et al., 1989; Rubanyi and Vanhoutte, 1985), whereas $EDCF_2$ is a cyclooxygenase product. Another EDCF, which is dependent on cyclooxygenase and TxA_2 synthetase, is released from aged rat aortic endothelium (Koga et al., 1989). In addition, superoxide radicals released from the endothelium can also act as EDCFs (Vanhoutte and Katusic, 1988). This possibility suggests an intriguing alternative explanation for the reduction in sensitivity to U-46,619 after TxA_2 synthetase inhibition. Mehta et al. (1988) demonstrated that TxA_2 synthetase inhibition reduces the release of oxygen-derived free radicals from neutrophils in the presence of platelets. Since oxygen-derived free radicals can activate the TxA_2/PGH_2 receptor pathway (Vanhoutte and Katusic, 1988; Auch-Schwelk et al., 1990). It is tempting to speculate that TxA_2 synthetase inhibition may reduce the response of the aortic rings to the TxA_2/PGH_2 mimetic by reducing oxygen-derived free radical generation. However, these studies do not provide any direct evidence for this hypothesis.

Some consequences of these findings require further work. First, platelet aggregation releases TxA_2 which

contributes to vasoconstriction of coronary arteries (Houston et al., 1986). Our findings indicate that this vasoconstriction may be augmented in regions of endothelial damage. Indeed, constrictor responses of iliac vessels to U-46,619 are increased in a model of advanced atherosclerosis in monkeys (Lopez et al., 1989). Second, the considerable variability in response to U-46,619 between species, between arterial beds in the same species, and even between the same vessels in the same species (Burke et al., 1983; Toda, 1984; Keith and Salama, 1987) may be explained in part by the extensive modulation of the response, both by EDRF which attenuates vasoconstriction and by release of TxA_2 which potentiates it.

Table 2-1. Effects of endothelium and of addition of UK-38,485 to supernatant fluid ex vivo or three days of administration of UK-38,485 in vivo on the ED_{50} responses of thoracic aortic rings to U-46,619.

Drug Administration	Number of rings	Endothelium intact	Endothelium removed	Effects of endothelium
		Series 1:		
None	13/14	$6.54\pm3.02\times10^{-9}$	$4.78\pm2.14\times10^{-10}$	$p<0.05$
Oxyhemoglobin	4	$6.63\pm0.38\times10^{-11}$	$5.13\pm0.18\times10^{-11}$	NS
		Series 2:		
Ex vivo:				
Vehicle	6	$5.65\pm2.99\times10^{-9}$	$1.17\pm0.43\times10^{-9}$	$p<0.05$
UK-38,485	6	$1.61\pm0.43\times10^{-8}$ *	$5.44\pm2.94\times10^{-9}$ *	$p<0.05$
		Series 3:		
In vivo:				
Vehicle	8/5	$2.18\pm0.51\times10^{-9}$	$3.40\pm0.18\times10^{-10}$	$p<0.05$
UK-38,485	8	$2.93\pm0.16\times10^{-9}$	$1.65\pm0.48\times10^{-9}$ *	$p<0.05$

Mean ± SEM data for ED_{50} values for development of force with U-46,619 (M) added to fluid bathing thoracic aortic rings. Effects of UK-38,485 compared to vehicle using Wilcoxon signed-rank or Mann-Whitney U tests: *, $p<0.05$.

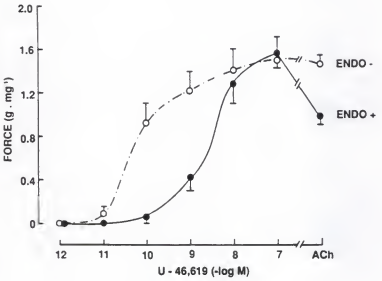

Figure 2-1. Dose-response relationships for U-46,619 generating force by aortic rings with and without endothelium.

Mean ± SEM values from Series 1. Rings were studied with endothelium removed (n = 14; open circles and broken lines) or endothelium intact (n = 13; solid circles and continuous lines). The effects of ACh (10^{-6} M) during administration of U-46,619 (10^{-7} M) are also shown.

Table 2-2. Release of immunoreactive TxB_2 into supernatant fluid bathing thoracic aortic rings with endothelium intact by U-46,619: effects of UK-38,485.

Drug Administration	Number of rings	TxB_2 ($pg \cdot ml^{-1}$) Before	After U-46,619	Effect of U-46,619
		Series 1:		
None	13	147 ± 12	211 ± 26	$p<0.05$
		Series 2:		
Ex vivo:				
Vehicle	6	59 ± 8	84 ± 13	$p<0.05$
UK-38,485	5	66 ± 8	75 ± 11	NS
		Series 3:		
In vivo:				
Vehicle	4	449 ± 252	556 ± 239	NS
UK-38,485	4	32 ± 21	11 ± 10 [a]	NS

Mean ± SEM values. Effects of UK-38,485 compared to vehicle using unpaired t-tests: a, $p<0.05$.

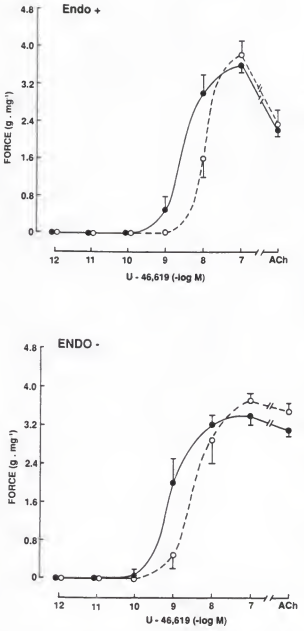

Figure 2-2. Dose-response relationships for U-46,619 generating force in aortic rings with and without endothelium: TxA_2 synthetase inhibitor administered ex vivo.

Mean ± SEM values from Series 2. Rings were studied during addition of vehicle (n = 6; solid circles and continuous lines) or UK-38,485 (10^{-6} M; n = 6; open circles and broken lines) to the supernatant fluid. The response to ACh (10^{-6}) during administration of U-46,619 (10^{-7} M) is shown. Responses of endothelium-intact vessels are shown in panel A and endothelium-denuded vessels in panel B.

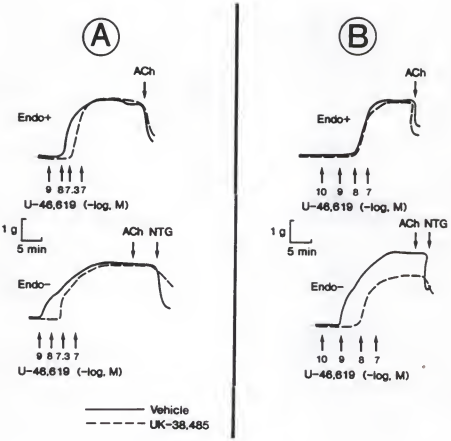

Figure 2-3. Representative dose-response relationships for U-46,619 generating force in aortic rings with and without endothelium: TxA_2 synthetase inhibitor administered ex vivo and in vivo.

Results from individual experiments from Series 2 (panel A) and Series 3 (panel B). In panel A, force records are shown for rings that had received vehicle (continuous lines) or UK-38,485 (10^{-6} M; broken lines) added to the bath. In panel B, force records are shown for rings from animals that had been pretreated for 3 days with vehicle (continuous lines) or with UK-38,485 (broken lines). The responses to ACh (10^{-6} M) or NTG (10^{-8}) during administration of U-46,619 are shown.

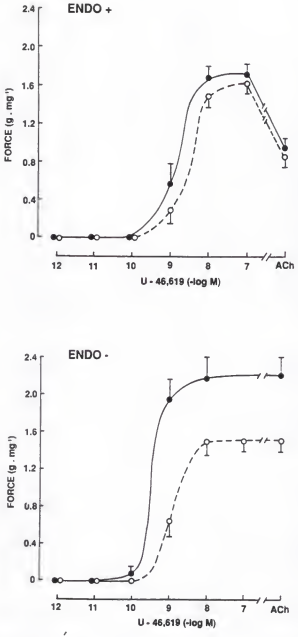

Figure 2-4. Dose-response relationships for U-46,619 generating force in aortic rings with and without endothelium: TxA_2 synthetase inhibitor administered in vivo.

Mean ± SEM values from Series 3. Animals were pretreated for 3 days with vehicle (n = 8; solid circles and continuous lines), or with UK38,485 (n = 8; open circles and broken lines). The response to ACh (10^{-6} M) during administration of U-46,619 (10^{-7} M) is shown. Responses of endothelium-intact vessels are shown in Panel A and endothelium-denuded vessels in Panel B.

CHAPTER 3
MECHANISM OF VASOCONSTRICTION OF RAT KIDNEYS WITH THE THROMBOXANE A_2 MIMETIC U-46,619

Introduction

Recently, TxA_2 has been found to contribute to renal vasoconstriction in a number of physiological states. Thus, TxA_2 is released by the kidney during infusion of angiotensin II (Wilcox and Welch, 1990a) and contributes to the increases in systemic and RVRs (Welch and Wilcox, 1988b; Wilcox and Welch, 1990b). Likewise, TxA_2 is released from the kidney by hyperchloremia (Wilcox et al., 1985) and contributes to the vasoconstriction of the pre- and post-glomerular arterioles (Bullivant et al., 1989). TxA_2 is also implicated in the vasoconstriction of pre-glomerular arterioles induced by activation of the tubuloglomerular feedback response (Welch and Wilcox, 1988a). This action of TxA_2 on tubuloglomerular feedback is independent of angiotensin II (Welch and Wilcox, 1990). In addition to these physiological actions, renal release of TxA_2 has also been implicated in the renal vasoconstriction that accompanies a number of pathophysiologic models of hypertension, nephritis, and nephropathy, including Dalh salt-sensitive nephritis (Yamashita et al., 1988), hypertension due to reduced renal mass (Purkerson et al., 1985), the early phase of

renovascular hypertension (Himmelstein and Klotman, 1989), autologous immune nephritis (Patrono et al, 1989), and ureteral occlusion (Nishikawa et al., 1977).

Infusions of TxA_2 itself (Cirino et al., 1990) or its stable analogue U-46,619 (Gerber et al., 1979; Feigen et al., 1977; Loutzenhiser et al., 1986) reduce the GFR and RBF of rat, dog, or pig kidneys. These actions may entail binding to the high-affinity receptors identified recently in renal glomeruli in the rat (Wilkes et al., 1989). However, there is considerable variability in the vascular response to TxA_2 or its mimetics (Keith and Salama, 1987) suggesting that there is an important degree of physiologic modulation. One modulator may be the sympathetic nervous system, since TxA_2 mimetics can release norepinephrine from meseneteric nerves (Jackson, 1985) or arteries (Okamura et al., 1988) and can increase the vascular responsiveness to norepinephrine (Okamura et al., 1988). Moreover, the pulmonary vasoconstriction that can occurs during infusion of TxA_2 (Cirino et al., 1990) and infusion of U-46,619 in conscious rats (Ahlstrom et al., 1990) may induce reflex cardiovascular responses. Therefore, one aim of the present studies is to evaluate the role of α-adrenoceptors in the renal vascular response to a TxA_2 mimetic.

TxA_2 mimetics can activate PLA_2 and stimulate PG release from coronary arteries <u>in vivo</u> (Mehta and Mehta, 1985) or aortic endothelial cells in culture (Hunt et al., 1989). Therefore, TxA_2-induced arachidonic acid release, with

metabolism to PGs, TxA_2, or other vasoactive agents such as leukotrienes, could be other pathways which modulate the response to TxA_2 receptor activation. Thromboxane A_2 is produced by interaction with endogenous leukotrienes to promote renal vasoconstriction during ureteral occlusion (Albrightson et al., 1987) or by immune glomerular injury (Rahman et al., 1987). Therefore, a second aim of these studies is to evaluate the role of TxA_2-induced PG, TxA_2, and leukotreine release in modulating renal vasoconstriction during infusions of U-46,619.

Methods

Animal Preparation

Studies were performed on male Sprague-Dawley rats weighing 150-250 grams maintained on a standard rat chow (Rodent Laboratories Chow 5001, Ralston Purina, St. Louis, MO). Anesthesia was induced with intraperitoneal Inactin (100 $mg \cdot kg^{-1}$: BYK Gulden, Konstanz, FRG). Animals were maintained at 37°C on a rodent operating table. After tracheostomy, one external jugular vein was cannulated for intravenous infusions, and one carotid artery was cannulated retrogradely with the tip of the catheter passed into the root of the aorta for intra-aortic drug infusions. The right femoral artery was cannulated for blood sampling and measurement of mean arterial blood pressure (MAP) from the

electrically-damped output of a pressure transducer (Model P23, Gould, Oxnard, CA). The other femoral artery was cleaned and encircled by an electromagnetic flow probe (Carolina Instruments Inc., Winston-Salem, NC) to measure femoral blood flow. The bladder was catheterized for collection of urine samples.

[^{3}H]-Inulin (2 g·dl^{-1}, Taylor Pharmacology, Decatur, IL), [^{14}C]-para-aminohippuric acid (PAH, 0.2 g·dl^{-1}, Merck, Sharp and Dohme, West Point, PA) and albumin (3 g·dl^{-1}, Sigma Chemicals, St. Louis, MO) in 0.154 M NaCl was given in a priming dose of 0.5 ml and as a maintenance infusion at 0.5 ml.100 g body wt^{-1}·h^{-1}. This protocol maintains a euvolemic state with a small reduction in hematocrit (Hct) (Welch and Wilcox, 1988a).

Thirty min after completion of surgery, there was a basal period of 30 min. Thereafter, the intra-aortic infusion was changed from 0.15 M NaCl to a solution of U-46,619 or its vehicle. After 10 min, a second (experimental) period was undertaken. Blood samples (0.6 ml) were obtained at the end of each clearance period and replaced with equal volumes of the albumin-in-saline solution used to deliver the renal clearance markers (see above). Measurements of MAP and femoral blood flow were undertaken at the beginning, midpoint, and end of each clearance period and the data averaged.

Experimental Protocols

Response to U-46,619 and blockade by SQ-29,548

The aim of this study was to investigate the dose-response effects of the TxA_2/PGH_2 mimetic, U-46,619, on MAP and on renal and femoral hemodynamics, and to assess whether any actions could be blocked by a specific competitive antagonist of the TxA_2/PGH_2 receptor, SQ-29,548. All rats were studied during two periods. For the dose-response studies, varying doses of U-46,619 (0.001, 0.01, 0.1, and 1 $\mu g \cdot kg^{-1} \cdot min^{-1}$) or its vehicle were infused during period 2 (Figure 3-1). Since the results of this study indicated that a dose of U-46,619 of 1 $\mu g \cdot kg^{-1} \cdot min^{-1}$ produced a half-maximal effect on renal hemodynamics without changing MAP or femoral blood flow consistently, this dose was selected for the subsequent studies. For group 1, the vehicle for U-46,619 was infused during period 2 via the intra-aortic cannula. For groups 2 and 3, U-46,619 (Upjohn Pharmaceuticals, Kalamazoo, MI) (Bundy, 1975) was dissolved in 1% ethanol, diluted with 0.15 M NaCl solution and infused during period 2 at 1 $\mu g \cdot kg^{-1} \cdot min^{-1}$ i.a. Group 3 received SQ-29,548 (8 $\mu g \cdot kg^{-1}$ and 8 $\mu g \cdot kg^{-1} \cdot h^{-1}$ i.v.) throughout. SQ-29,548 (Squibb Institute for Medical Research, Summit, NJ) (Ogletree et al., 1985; Darius et al., 1985) was dissolved in ethanol and diluted in 0.15 M NaCl solution.

Effects of drugs which antagonize α-adrenoceptors or leukotriene D_4/E_4 receptors or which inhibit cyclooxygenase or TxA_2 synthetase on renal and femoral vascular response to U-46,619

The aim of these studies was to define the role of α-adrenoceptors or leukotriene D_4/E_4 receptors or products of cyclooxygenase or TxA_2 synthetase on the hemodynamic response to U-46,619. Rats were studied during a basal period and during an intra-aortic infusion of U-46,619 as in protocol 1 above. Each animal received an agonist or inhibitor drug as a pretreatment or at completion of surgery (Table 3-1). Group 4 received the α-adrenoceptor antagonist phenoxybenzamine (PBZ). PBZ was dissolved in 0.15 M NaCl and given as an intravenous bolus injection of 300 $\mu g \cdot kg^{-1}$ and a maintenance infusion of 300 $\mu g \cdot kg^{-1} \cdot h^{-1}$. Group 5 received three daily intraperitoneal injections of the cyclooxygenase inhibitor indomethacin (5 $mg \cdot kg^{-1} \cdot d^{-1}$; Sigma Chemicals, St. Louis, MO). Indomethacin was dissolved in a 1 M Na_2CO_3 solution at pH 12.5, titrated to pH 8.5 with HCl and diluted in 0.15 M saline. Group 6 received three daily intraperitoneal injections of the TxA_2 synthetase inhibitor UK-38,485 (50 $mg \cdot kg^{-1} \cdot d^{-1}$; Pfizer Central Research, Groton, CT) (Cross et al., 1986; Parry et al., 1982). UK-38,485 was dissolved in 1 N NaOH at pH 12.5 and titrated with 1 N HCl to pH 8.5 at 100 $mg \cdot ml^{-1}$. Group 7 received the leukotriene D_4/E_4 receptor antagonist LY-163,443 (5 $mg \cdot kg^{-1}$; Lilly Research, Indianapolis, IN) (Fleisch et al., 1986; Fleisch et al.,

1988) at the completion of surgery and a maintenance infusion of 5 $mg \cdot kg^{-1} \cdot h^{-1}$ LY-163,443 was dissolved in 0.15 M NaCl.

Chemical Methods and Calculations

Sodium concentrations in plasma and urine were measured by flame photometry (IL Flame Photometer, Lexington, MA). The GFR was estimated from the clearance of [^{3}H]-Inulin and the RPF from the clearance of [^{14}C]-PAH (New England Nuclear, Boston, MA) with correction for quenching and cross-counting but without correction for renal extraction. The RBF was calculated from RPF and Hct and the RVR and femoral vascular resistance from the MAP and the RBF or femoral blood flow. The fractional excretion of sodium (FE_{Na}) was calculated from the urine-to-plasma concentration ratio for sodium divided by that for inulin.

The purification of the TxA_2 derivative, TxB_2 and PGI_2 derivative $6kPGF_{1\alpha}$ from urine and the details of the assay methods, together with its performance characteristics and validation have been published (Welch and Wilcox, 1988a; Welch et al., 1989). Briefly, aliquots (0.5 ml) of urine samples were spiked with approximately 100 $count \cdot min^{-1}$ of [^{3}H]-TxB_2 to assess the individual recovery of each sample. Aliquots were diluted with 0.5 ml of deionized water and adjusted to pH 3 with 1 N HCl. Samples were extracted with 8 ml of ethyl acetate. The organic phase was dried under N_2 and dissolved in 60 µl of acetone before loading onto a

silicone gel C-plate (Fischer Scientific, Springfield, NJ). The samples were extracted with 8 ml of methyl formate, centrifuged, dried under N_2, and dissolved in 500 µl of phosphate buffer (pH 7.4). A 50 µl aliquot of the purified material was used for calculation of recovery, and radioimmunoassays were performed on the remainder at 4°C using specific antisera (Sergen, Boston, MA). In preliminary studies, a dose of U-46,619 equivalent to the total administered to the rat was added to a 1 ml aliquot of rat urine. This did not effect the assays for $6kPGF_{1\alpha}$ or TxB_2. Since the drugs which were used altered the GFR which is a determinant of PG excretion, renal excretion data were all factored by GFR.

Statistics

Data are presented as the mean ± SEM. They were assessed by analysis of variance (ANOVA) and post-hoc hypotheses were tested by an unpaired t-test. Differences were considered significant if $p < 0.05$.

Results

The dose-response relationships between increasing doses of intravenously-infused U-46,619 and changes in systemic and renal hemodynamics are shown in Figure 3-2. It was clear that the renal hemodynamic change (RVR) occurred at a lower dose of infused U-46,619 than that required to increase

femoral vascular flow or MAP. Moreover, the fractional changes in renal hemodynamics were quantitatively much more impressive than in the other parameters. Since a dose of U-46,619 of 1 $\mu g \cdot kg^{-1} \cdot min^{-1}$ produced an approximately 50% reduction of GFR and doubling RVR (Figure 3-3) without major effects on MAP, HR, or FVR (Figure 3-4), this dose was selected for subsequent studies.

For series 1, the hemodynamic and renal responses to U-46,619, and its antagonism by SQ-29,548, are shown in Table 3-2. In the time control group, which received an infusion of the vehicle for U-46,619 during the second period (group 1), there was a small fall in MAP and rise in urine flow (UV), but other parameters remained stable. In contrast, where U-46,619 was infused during period 2 (group 2), ANOVA demonstrated that there was a small increase in the MAP but profound reductions averaging 48% in the GFR and RBF. Although the calculated RVR more than doubled, there were no consistent changes in femoral vascular resistance. All these effects of U-46,619 were fully blocked by concurrent infusions of SQ-29,548 (Figure 3-5).

The excretion of PGs and TxB_2 during series 1 are shown in Table 3-3, and PG and TxB_2 excretion factored by GFR are shown in Figure 3-6. No consistent changes from basal values was found in any either PG or TxB_2 with the infusion of vehicle or U-46,619 during period 2. In contrast, the infusion of U-46,619 lead to substantial increases in the excretion of both PGs and TxB_2 when factored by GFR. These

increases were abolished by concurrent infusion of SQ-29,548 (Figure 3-7).

The functional significance of the increased production of PGs and TxA_2 and the possible role of leukotreines C_4 and D_4 or activation of the sympathetic nervous system was assessed in series 2 (Table 3-4). The role of the sympathetic nervous system was studied in group 4. As shown in Figure 3-8, pretreatment with the long-duration α-adrenoceptor antagonist PBZ significantly blunted the effects of U-46,619 on GFR and RBF. The role of cyclooxygenase products was studied in group 5 (Figure 3-9). The three daily injections of indomethacin reduced the baseline MAP and RBF significantly, but other baseline parameters were not altered. After indomethacin the U-46,619-induced reductions in GFR and RBF were not significantly changed; they averaged 53% and 40%, respectively compared to 48% and 48% for U-46,619 alone in group 2. In contrast to the lack of consistent effects of indomethacin on renal hemodynamic changes with U-46,619, it did prevent the increase in UV. The effects of TxA_2 synthetase inhibitor pretreatment on the response to U-46,619 were examined in group 6 (Figure 3-9). The three daily injections of UK-38,485 reduced the baseline femoral vascular resistance significantly and increased the baseline UV and FE_{Na}. UK-38,485 significantly blunted the reductions in GFR and RBF and rise in RVR during infusion of U-46,619. The effects of the competitive antagonist of leukotriene D_4/E_4 receptors was examined in group 7

(Figure 3-9). In this group, the infusion of LY-163,443 reduced the basal GFR significantly ($p<0.05$) by 21%. However, despite this reduction, LY-163,443 infusion blunted significantly the reductions in GFR and RBF and the increases in RVR during infusion of U-46,619.

The effect of some of the drugs used in series 2 on excretion of PGs and TxA_2 are shown in Table 3-5, and the PG and TxA_2 excretion factored by GFR are shown in Figure 3-10 and Table 3-6. The only significant change from basal levels with infusion of vehicle or U-46,619 during period 2 occurred as an increase in $6kPGF_{1\alpha}$ when pretreated with LY-163,443 (Table 3-5). However, when factored by GFR, indomethacin pretreatment reduced the basal excretion of PGs by greater than 75% and blunted the increases in PG excretion during infusion of U-46,619. As anticipated, UK-38,485 pretreatment reduced the basal TxB_2/GFR by 89%. However, there was also a significant reduction in the basal rate of excretion of $6kPGF_{1\alpha}$/GFR. In this group, the U-46,619-induced increases in the excretion of PGE_2 or $6kPGF_{1\alpha}$ were not modified consistently, but the rise in TxB_2 was severely attenuated. During infusion of LY-163,443 (group 7), neither the basal rates of excretion nor the U-46,619-induced increases in excretion of $6kPGF_{1\alpha}$ and TxB_2 per unit GFR were altered consistently.

Discussion

The main new findings of this study are that the vascular resistance in the kidney is far more responsive to a TxA_2/PGH_2 receptor agonist than is the resistance in the femoral vessels. Renal vasoconstriction and reduction in GFR are accompanied by increased excretion of PGs and TxB_2, factored by GFR, and they are substantially blunted or prevented by drugs which inhibit TxA_2 synthesis, as well as by activation of leukotreine D_4/E_4 or alpha adrenergic receptors. In contrast, these renal hemodynamic changes are maintained after pretreatment with a dose of indomethacin sufficient to reduce PG excretion by 75%.

The present findings confirm that intrarenal infusions of TxA_2 or its stable mimetic reduce GFR and/or RBF (Feigan et al., 1977; Gerber et al., 1979; Cirino et al., 1990). The finding in the present series of proportionate reductions in GFR and RBF during infusion of U-46,619 is consistent with proportionate increases in pre- and post-glomerular vascular resistances recorded during infusion of U-46,619 in the Munich-Wistar rat (Baylis, 1987).

At the dose of U-46,619 tested, the RVR and GFR were far more sensitive and responsive than were the MAP and FVR. Moreover, our previous studies showed that infusion of U-46,619 at a dose of only 0.01 $\mu g \cdot kg^{-1} \cdot min^{-1}$ increased the tubuloglomerular feedback response (Welch and Wilcox, 1990)

and reduced renal renin secretion (Welch et al., 1989). This dose of U-46,619 is only 1% of that required to have noticable systemic vascular effects and highlights the extreme sensitivity and responsiveness of the kidney to infused U-46,619. Indeed, engagement of tubuloglomerular feedback may underlie the extreme sensitivity of the renal circulation to U-46,619.

Recent studies with rat glomerular membranes have identified a high-affinity binding site for radiolabelled SQ-29,548, which is displaced by U-46,619 (Wilkes et al., 1989). The observation that the fall in RBF and GFR with U-466,19 is antagonized in full by SQ-29,548 suggests that U-46,619 activates TxA_2/PGH_2 receptors in vivo to initiate renal vasoconstriction. However, the studies of PG and TxA_2 excretion and of the effects of inhibitors or antagonists demonstrate a widespread and profound modulation of this response.

U-46,619 can activate PLC and PLA_2 and releases TxA_2 and PGI_2 from platelets, blood vessels, endothelial cells, aortic rings, and glomerular mesangial cells (Hunt et al., 1989; Mehta and Roberts, 1983; Mené et al., 1988; Pollock et al., 1984; see Chapter 2). These actions of U-46,619 to increase PG production have been confirmed in vivo (Mehta et al., 1984). In this series, the interpretation of changes in PG excretion was complicated by the sharp reductions in GFR. However, U-46,619 did increase the excretion of PGs and TxB_2 factored by GFR. Moreover, in a previous series, infusions

of U-46,619 in the rat, at a dose well below that required to change renal hemodynamics, increased excretion of PGE_2, $6kPGF_{1\alpha}$ and TxB_2 (Welch et al., 1989). Therefore, we concluded that U-46,619 probably increased renal PG production, and we proceeded to investigate the functional consequences by using drugs which inhibit production of PGs and/or TxA_2. We found no significant effects of indomethacin pretreatment on the renal hemodynamic response to U-46,619 despite profound reductions in PG and TxB_2 excretion. This confirms the slight or absent effects of cyclooxygenase inhibition seen previously. Thus, in one study, indomethacin increased modestly the renal vasoconstriction produced by an intrarenal infusion of a prostaglandin endoperoxide analogue (Gerber et al., 1979). However, in other studies, indomethacin did not modify the vasoconstrictor action of U-46,619 on the mesenteric vessels in the cat (Lipton et al., 1986) or the renal vessels of the dog (Feigan et al., 1977) or pig (Cirino et al., 1990). However, the conclusion that PGs have little effect on the response to U-46,619 conflicts with the evidence of PG release. Therefore, we investigated an alternative hypothesis that there was a balanced release of PGI_2 and TxA_2 and that non-selective inhibition of vasodilator and vasoconstrictor PG synthesis with a cyclooxygenase inhibitor would mask effects of TxA_2. Indeed, a more selective reduction in TxA_2 excretion induced by UK-38,485 led to marked blunting of the renal vasoconstrictor action of U-46,619. This extends our preliminary findings in

rat thoracic aortic rings where U-46,619 increased local vascular synthesis of PGI_2 and TxA_2, and the contractile response to U-46,619 was unchanged by indomethacin but blunted by UK-38,485 (Folger et al., 1989). These data are consistent with the hypothesis that U-46,619 releases TxA_2 from vascular tissue in the kidney and that this release of endogenous TxA_2 contributes to the renal vasoconstriction. However, alternative explanations should be considered. The 3 days of pretreatment with UK-38,485 increased platelet PGH_2 levels and thereby increased the synthesis of vascular PGI_2 making the vessel less responsive to contractile agonists. However, our data show that after UK-38,485 pretreatment, the basal excretion of $6kPGF_{1\alpha}$ was actually reduced while the rise in excretion with U-46,619 was maintained. This argues against an important degree of shunting of endoperoxide metabolism towards PGI_2 synthesis during UK-38,485 administration in these studies. Pretreatment with UK-38,485 may have altered TxA_2/PGH_2 receptor number or affinity. Indeed, platelet TxA_2/PGH_2 receptors are rapidly down-regulated by exposure to a TxA_2/PGH_2 mimetic. It is, however, hard to predict the effects of UK-38,485 on TxA_2/PGH_2 receptor number. Thus, although UK-38,485 should reduce TxA_2 generation, any increase in PGH_2 production might be sufficient to counteract this or even to down-regulate vascular TxA_2/PGH_2 receptors. However, for reasons discussed above, the data do not support a major re-direction of endoperoxide metabolism by UK-38,485 in these studies,

although PGH_2 generation could not be assessed. Finally, endogenous TxA_2 generation promotes norepinephrine release from sympathetic nerve terminals. As discussed below, enhanced norepinephrine release may have contributed to the renal vasoconstrictor response to infusion of U-46,619 in these studies. Therefore any reduction in release induced by UK-38,485 pretreatment may have contributed to the blunted vasoconstrictor response to U-46,619 observed.

TxA_2-induced phospholipase activation may also generate other vasoactive arachidonic acid metabolites besides TxA_2. Indeed, O'Keefe et al. (1985) demonstrated that arachidonic acid releases an undefined vasoconstrictor metabolite in addition to TxA_2 from the lung. The present study indicates that leukotrienes participate in renal vasoconstriction with U-46,619. There are specific receptors for leukotriene C_4 in rat glomeruli (Ballermann et al., 1985). Infusion of leukotriene C_4 reduces RBF in the rat (Badr et al., 1987). Both leukotriene C_4 and leukotriene D_4 are vasoconstrictors in the isolated rat kidney (Rosenthal and Pace-Asciak, 1982) and can contract rat glomerular mesangial cells in culture (Simonson and Dunn, 1986). Our findings that the potent and specific leukotriene D_4/E_4 receptor antagonist, LY-163,443, blunts the renal vasoconstrictor response to U-46,619 suggest that leukotriene formation contributes to TxA_2-induced renal vasoconstriction in the intact rat kidney. However, the source of the leukotriene D_4 and/or leukotriene E_4 that are implicated in this response is quite uncertain. The

synthesis of leukotriene B_4 by 5-lipoxygenase activity in isolated glomeruli has been reported in one early study (Cattell et al., 1984), but others have not been successful in confirming this finding which might be ascribed to leukocytes trapped in the glomerular capillaries. However, glomeruli do possess the enzymes required to process leukotriene A_4 to leukotriene C_4 and leukotriene E_4. (Sraer et al., 1986). Therefore, leukotriene A_4 may have been generated elsewhere, perhaps in leucocytes traversing the renal vessels or in endothelial cells within the renal vessels in response to U-46,619, and it may have been processed in renal vessels or glomeruli to the vasoconstrictor metabolites, leukotriene D_4 and leukotriene E_4.

TxA_2 (Trachte and Stein, 1988) or TxB_2 (Okamura et al., 1988) can increase norepinephrine release from sympathetic nerve terminals located in vascular tissue. Moreover, inhibition of TxA_2 synthesis in the perfused rat mesentery has been shown to impair noradrenergic sympathetic transmission (Jackson et al., 1988; Jackson, 1985). Therefore, we assessed the effects of α-receptor blockade on U-46,619-induced vasoconstriction. In the present study, PBZ given in a dose that we found previously to blunt the pressor response to norepinephrine in the rat by >90% (Luft et al., 1989) severely attenuated the renal vasoconstrictor response to U-46,619. This observation suggests that infusion of U-46,619 increases norepinephrine release which contributes

to the renal vasoconstriction. It is unclear whether this represents a direct action of U-46,619 on sympathetic nerves, a central action in the brain, or stimulation of cardiovascular reflexes. In conscious SHR, intracerebroventricular administration of U-46,619 induces hypertension (Sirén et al., 1985), but it has no effect on BP in anesthetized normotensive rats. These findings suggest an equivocal role for TxA_2 in central cardiovascular control. Recently, it was found that activation of the sympathetic nervous system is an important mechanism contributing to hypertension during prolonged infusion of U-46,619 into conscious rats (Ahlstrom et al., 1990). This long-term thromboxane-dependent hypertension is blunted by PBZ and anesthesia.

The present results demonstrate a remarkable degree of enforcement of U-46,619-induced renal vasoconstriction by recruitment of additional vasoconstrictor mechanisms that include endogenous release of TxA_2, activation of leukotriene D_4/E_4 receptors, and activation of α-adrenoceptors. This observation may explain the extreme sensitivity of the renal circulation to U-46,619.

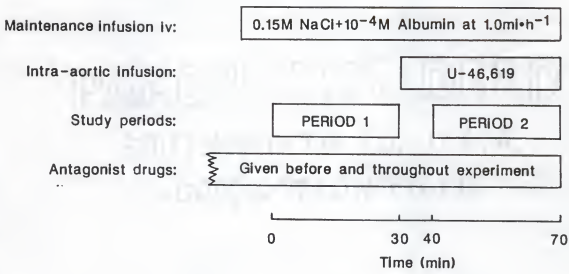

Figure 3-1. Protocol for U-46,619 infusions.

Table 3-1. Experimental groups for infusion of U-46,619 with administration of cyclooxygenase or thromboxane synthetase inhibitors or α-adrenoceptor, leukotriene D_4/E_4 receptor or TxA_2/PGH_2 receptor antagonists.

Group	Number of rats	Drug	Dose	Pharmacological action
1	10	Vehicle		
2	8	SQ-29,548 (SQ)	8 $mg \cdot kg^{-1} \cdot hr^{-1}$	Competitive antagonist of TxA_2/PGH_2 receptors
3	6	Indomethacin (Indo)	5 $mg \cdot kg^{-1} \cdot d_{-1} x3$	Inhibits cyclooxygenase
4	7	UK-38,485 (UK)	50 $mg \cdot kg^{-1} \cdot d^{-1} x3$	Inhibits thromboxane synthetase
5	6	LY-163,443 (LY)	5 $mg \cdot kg^{-1} \cdot hr^{-1}$	Competitive antagonist of leukotriene D_4/E_4 receptors
6	7	Phenoxybenzamine (PBZ)	300 $\mu g \cdot kg^{-1}$	Noncompetitive antagonist of α-adrenoceptors

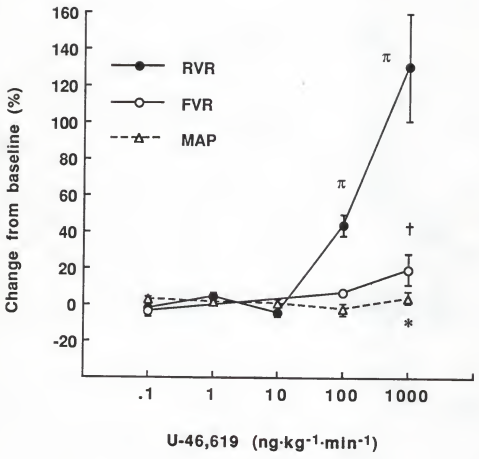

Figure 3-2. Dose-response relationships for TxA_2 mimetic.

Mean ± SEM values for changes in baseline per cent of MAP, FVR, or RVR during infusions of U-46,619 (0, 0.1, 1.0, 10, 100 ng·kg^{-1}·min^{-1} i.a.). *, $p<0.05$; †, $p<0.01$; π, $p < 0.001$.

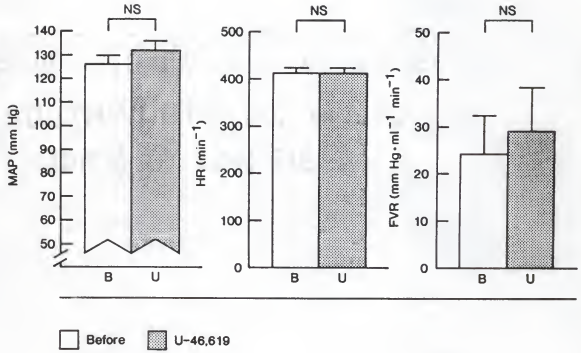

Figure 3-3. Effects of TxA_2 mimetic on systemic hemodynamics.

Mean ± SEM values for mean arterail blood pressure (MAP), heart rate (HR), or femoral vascular resistance (FVR) during infusion of vehicle (B, before; n = 10 or 6 for FVR) or U-46,619 (U, n = 10 or 6 for FVR) (1 $\mu g \cdot kg^{-1} \cdot min^{-1}$ i.a.).

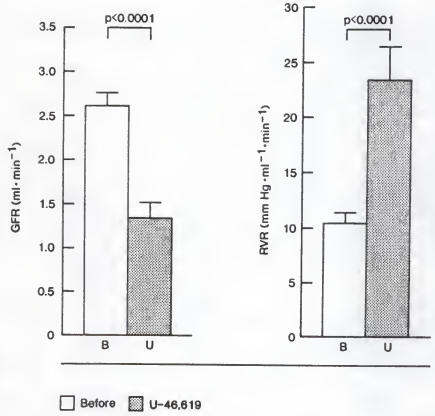

Figure 3-4. Effects of TxA_2 mimetic on renal function.

Mean ± SEM values for GFR or RVR during infusion of vehicle (B, before; n = 10) or U-46,619 (U, n = 10) (1 $\mu g \cdot kg^{-1} \cdot min^{-1}$ i.a.).

Table 3-2. Response of blood pressure, femoral vascular resistance, renal hemodynamics, and renal excretion of fluid and sodium to U-46,619: effects of SQ-29,548, a TxA_2/PGH_2 antagonist.

	MAP (mmHg)	FVR ($mmHg \cdot ml^{-1} \cdot min^{-1}$)	GFR ($ml \cdot min^{-1}$)	RBF ($ml \cdot min^{-1}$)	RVR ($mmHg \cdot ml^{-1} \cdot min^{-1}$)	UV ($\mu l \cdot min^{-1}$)	FE_{Na} (%)
Group 1 (n=7):							
1. Basal	128±2	29±4	2.69±0.18	12.8±0.7	10±1	9±3	0.4±0.3
2. Vehicle	124±2	28±4	2.64±0.27	13.2±1.1	10±1	21±6	1.0±0.4
*	-4±1	-1±1	-0.05±0.15	+0.3±0.8	0±0	+11±4	+0.6±0.3
Group 2 (n=10):							
1. Basal	126±4	35±8	2.61±0.17	12.7±1.0	10±1	9±2	0.1±0.0
2. U-46,619	132±5	41±6	1.35±0.17	6.5±0.8	23±3	21±6	0.7±0.2
*	+6±4	+7±3	-1.26±0.13	-6.2±0.8	+13±3	+12±5	+0.7±0.2
Group 3 (n=8):							
1. SQ-29,548	109±4	29±2	2.25±0.21	12.1±1.2	10±1	4±1	0.0±0.0
2. SQ + U	108±6	28±2	2.40±0.21	11.8±1.4	10±1	13±3	0.4±0.2
*	-1±3	-1±1	+0.15±0.23	-0.5±1.5	+1±1	+9±3	+0.4±0.2
By ANOVA:							
Effects of U-46,619:	p<0.05	NS	p<0.0001	p<0.0001	p<0.001	NS	NS
Effects of SQ on response to U-46,619:	p<0.05	NS	p<0.0001	p<0.001	p<0.0005	NS	NS

Mean ± SEM, data from rats of series 1. U-46,619, or equivalent vehicle, was infused at 1 $\mu g \cdot kg^{-1} \cdot min^{-1}$ during period 2. SQ-29,548 (SQ; 8 $mg \cdot kg^{-1}$ and 8 $mg \cdot kg^{-1} \cdot h^{-1}$) or vehicle were infused throughout. *, mean ± SEM differences between periods 1 and 3.

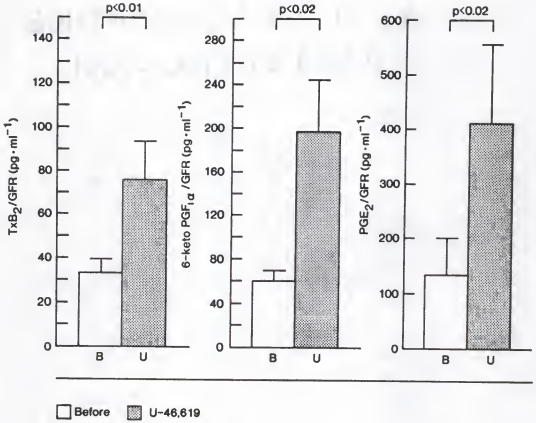

Figure 3-5. Effects of TxA_2 mimetic on PG excretion per unit GFR.

Mean ± SEM values for TxB_2, $6kPGF_{1\alpha}$, or PGE_2 factored by GFR during infusion of vehicle (B, before; n = 10) or U-46,619 (U, n = 10) (1 $\mu g \cdot kg^{-1} \cdot min^{-1}$ i.a.).

Table 3-3. PG excretion with U-46,619: effects of TxA_2/PGH_2 receptor antagonist.

	PGE_2 ($pg \cdot min^{-1}$)	$6kPGF_{1\alpha}$ ($pg \cdot min^{-1}$)	TxB_2 ($pg \cdot min^{-1}$)
Group 1 (n=7):			
1. Basal	311± 82	245±24	39± 9
2. Vehicle	319±159	252±35	38±10
*	+8±84	+7±23	-1±2
Group 2 (n=10):			
1. Basal	295±129	146±13	80±11
2. U-46,619	414±135	236±61	92±22
*	+119±162	+90±59	+12±17
Group 3 (n=8):			
1. SQ	114±33	80±12	53± 7
2. SQ+U-46,619	82±19	78±24	52±10
*	-33±31	-2±22	0±8
By ANOVA:			
Effects of U-46,619:	NS	NS	NS
Effects of SQ on response to U-46,619:	NS	NS	NS

Mean ± SEM values data from rats of series 1. U-46,619 or an equivalent vehicle was infused intravenously at 1 $\mu g \cdot kg^{-1} \cdot min^{-1}$) during period 2. SQ, SQ-29,548 (SQ: 8 $mg \cdot kg^{-1}$and 8 $mg \cdot kg^{-1} \cdot h^{-1}$) or its vehicle was infused throughout. *, mean ± SEM difference between periods 1 and 2.

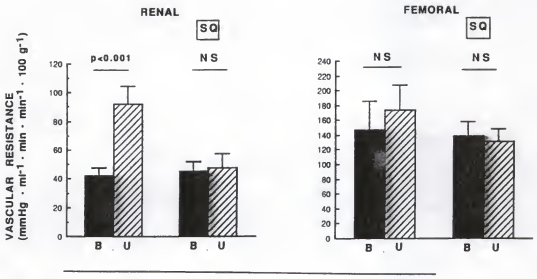

Figure 3-6. Comparison of renal and femoral vascular resistance to TxA_2 mimetic.

Mean ± SEM values for vascular resistance during infusion of vehicle (B, before) or U-46,619 (U) (1 $\mu g \cdot kg^{-1} \cdot min^{-1}$ i.a., n = 10 or 6 for FVR) after pretreatment with SQ (SQ-29,548, 8 $mg \cdot kg^{-1}$ and 8 $mg \cdot kg^{-1} \cdot hr^{-1}$, n = 8)

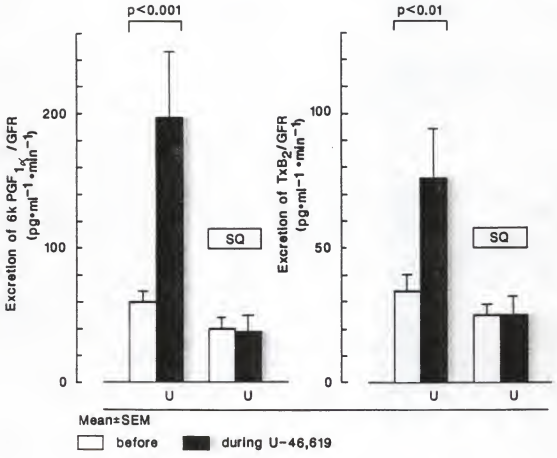

Figure 3-7. Renal prostacyclin and TxA_2 excretion factored by GFR with TxA_2 mimetic: effects of TxA_2/PGH_2 antagonist.

Mean ± SEM values for $6k\dot{P}GF_{1\alpha}$ or TxB_2 factored by GFR during infusion of vehicle or U-46,619 (U) (1 $\mu g \cdot kg^{-1} \cdot min^{-1}$ i.a., n = 10) after pretreatment with SQ (SQ-29,548, 8 $mg \cdot kg^{-1}$ and.8 $mg \cdot kg^{-1}.hr^{-1}$, n = 8).

Table 3-4. Effects of an α-adrenoceptor antagonist, an inhibitor of cyclooxygenase or thromboxane synthetase, or a leukotriene D_4/E_4 receptor antagonist on the response to U-46,619.

	MAP (mmHg)	FVR	GFR	RBF	RVR	UV	FE_{Na} (%)
		($mmHg \cdot ml^{-1} \cdot min^{-1}$)	($ml \cdot min^{-1}$)	($ml \cdot min^{-1}$)	($mmHg \cdot ml^{-1} \cdot min^{-1}$)	($\mu l \cdot min^{-1}$)	
Group 2 (n=10):							
1. Basal	126±4	35±8	2.61±0.17	12.7±1.0	10±1	9±2	0.1±0.0
2. U-46,619	132±5	41±6	1.35±0.17	6.5±0.8	23±3	21±6	0.7±0.2
*	+6±4	+7±3	-1.26±0.13	-6.2±0.8	+13±3	+12±5	+0.7±0.2
Group 4 (n=7):							
1. Basal + PBZ	99±4	15±3	2.59±0.23	11.2±0.6	9±0	8±4	0.1±0.1
2. PBZ + U-46,619	113±7	16±2	2.15±0.21	9.2±0.8	13±1	13±8	0.5±0.4
*	+14±3	+1±1	-0.45±0.16	-2.0±0.8	+4±1	+6±4	+0.3±0.3
Group 5 (n=6):							
1. Basal + Indo	108±5	28±3	2.24±0.17	9.9±0.9	11±1	7±3	0.1±0.1
2. Indo + U-46,619	103±8	30±4	1.05±0.18	5.0±0.6	23±3	3±1	0.1±0.1
*	-6±8	+2±3	-1.19±0.33	-4.0±0.5	+11±3	-4±2	0.0±0.1
Group 6 (n=7):							
1. Basal + UK	120±4	20±1	2.58±0.14	12.9±0.7	9±0	17±3	0.3±0.1
2. UK + U-46,619	126±5	23±1	2.04±0.07	10.1±0.7	12±0	34±9	1.7±0.6
*	+6±3	+3±1	-0.54±0.08	-2.7±0.4	+3±0	+17±7	+1.4±0.5
Group 7 (n=6):							
1. Basal + LY	112±5	----	2.07±0.13	8.9±0.5	13±1	16± 8	----
2. LY + U-46,619	110±7	----	1.74±0.12	9.1±0.8	12±0	28±14	----
*	-2±3	----	-0.33±0.08	+0.2±0.7	0±1	+11±10	----
Effect of drugs on response to U-46,619 by ANOVA:							
Group 4 (PBZ)	NS	NS	p<0.05	p<0.05	p<0.05	NS	NS
Group 5 (Indo)	NS	NS	NS	NS	NS	p<0.05	NS
Group 6 (UK)	NS	NS	p<0.02	p<0.02	p<0.004	NS	NS
Group 7 (LY)	NS	--	p<0.05	p<0.05	p<0.05	NS	--

Mean ± SEM data from series 2. U-46,619 (UK: 10 $\mu g \cdot kg^{-1} \cdot min^{-1}$); Phenoxybenzamine (PBZ; 300 $\mu g \cdot kg^{-1}$ and $\mu g \cdot kg^{-1} \cdot min^{-1}$); Indomethacin (Indo; 5 $mg \cdot kg^{-1} \cdot d^{-1}$ x 3); UK-38,485 (UK; 50 $mg \cdot kg^{-1} \cdot d^{-1}$x 3); LY-163,443 (LY; 5 $\mu g \cdot kg^{-1}$ and 5 $\mu g \cdot kg^{-1} \cdot min^{-1}$). *, mean ± SEM difference between periods 1 and 2.

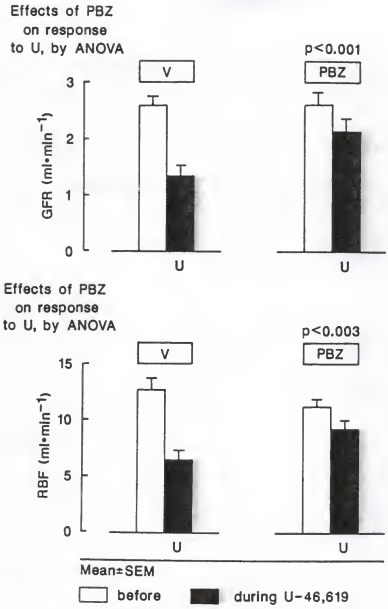

Figure 3-8. GFR and RBF with TxA_2 mimetic: effects of α-adrenoceptor blockade.

Mean ± SEM values for GFR or RVR during infusion of vehicle or U-46,619 (U) (1 $\mu g \cdot kg^{-1} \cdot min^{-1}$ i.a.) after pretreatment with vehicle (V, n = 10) or phenoxybenzamine (PBZ, 300 $\mu g \cdot kg^{-1}$, n = 7).

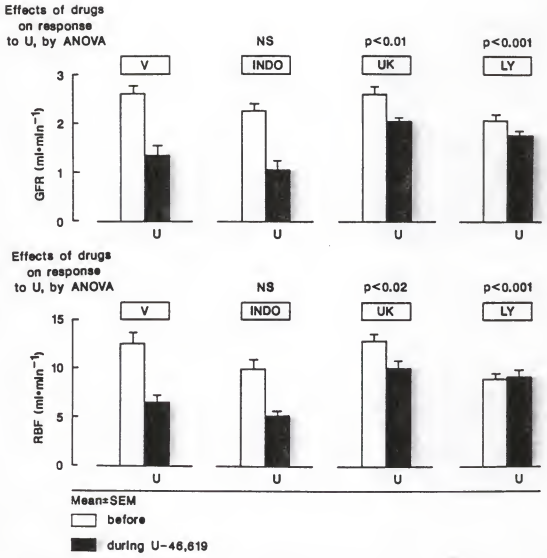

Figure 3-9. GFR and RBF with TxA_2 mimetic: effects of inhibition of cyclooxygenase or TxA_2 synthetase, or leukotriene D_4/E_4 receptor antagonist.

Mean ± SEM values for GFR or RVR during infusion of vehicle or U-46,619 (U) (1 $\mu g \cdot kg^{-1} \cdot min^{-1}$ i.a.) after pretreatment with vehicle (V, n = 10), indomethacin (indo, 5 $mg \cdot kg^{-1} \cdot d^{-1}$ x 3, n = 6), UK (UK-38,485, 50 $mg \cdot kg^{-1} \cdot d^{-1}$ x 3, n = 7), or LY (LY-163,443, 5 $mg \cdot kg^{-1} \cdot hr^{-1}$, n = 6).

Table 3-5. Effects of α-adenoceptor antagonist, inhibitors of cyclooxygenase or thromboxane synthetase, or antagonist of leukotriene C_4/D_4 receptor on PG excretion during infusion of TxA_2/PGH_2 mimetic.

	PGE_2 ($pg \cdot min^{-1}$)	$6kPGF_{1\alpha}$ ($pg \cdot min^{-1}$)	TxB_2 ($pg \cdot min^{-1}$)
Group 2 (n=10):			
1. Basal	295±129	146±13	80±11
2. U-46,619	414±134	236±61	92±22
*	+120±162	+90±59	+12±17
Group 4 (n=7):			
1. PBZ	462±92	156±48	93±29
2. PBZ + U-46,619	396±69	159±34	79±28
*	-66±62	+3±26	-13±16
Group 5 (n=6):			
1. Indo	27±2	0±0	16±5
2. Indo + U-46,619	26±7	0±0	8±3
*	+0±6	0±0	-8±4
Group 6 (n=7):			
1. UK	221±46	64±16	9±2
2. UK + U-46,619	409±70	147±40	13±4
*	+188±99	+83±33	+4±5
Group 7 (n=6):			
1. LY	ND	207± 38	52±11
2. LY + U-46,619	ND	697±318	111±37
*		+490±326	+59±37
Effect of drugs on response to U-46,619 by ANOVA:			
Group 4 (PBZ)	NS	NS	NS
Group 5 (Indo)	NS	NS	NS
Group 6 (UK)	NS	NS	NS
Group 7 (LY)	--	$p<0.02$	NS

Mean ± SEM. Data from series 2. U-46,619 was infused during period 2 at 1 $\mu g \cdot kg^{-1} \cdot min^{-1}$; PBZ, phenoxybenzamine; Indo, Indomethacin; UK, UK-38,485; LY, LY-163,443. *, mean ± SEM difference between periods 1 and 2.

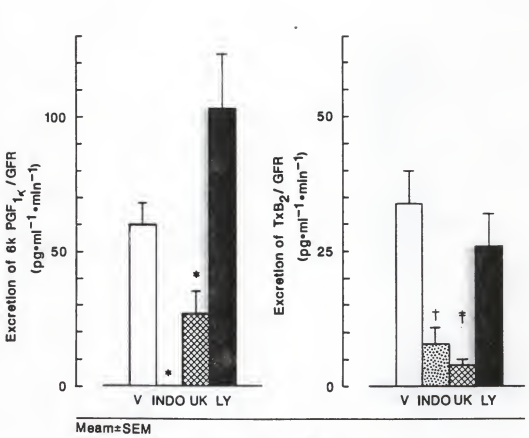

Figure 3-10. Basal prostacyclin and TxA_2 excretion: effects of inhibition of cyclooxygenase, TxA_2 synthetase, or leukotriene D_4/E_4 receptors.

Mean ± SEM values for $6kPGF_{1\alpha}$ or TxB_2 factored by GFR during infusion of U-46,619 (1 $\mu g \cdot kg^{-1} \cdot min^{-1}$ i.a.) after pretreatment with vehicle (V, n = 10), indomethacin (indo, 5 $mg \cdot kg^{-1} \cdot d^{-1}$ x 3, n = 6), UK (UK-38,485, 50 $mg \cdot kg^{-1} \cdot d^{-1}$ x 3, n = 7), or LY (LY-163,443, 5 $mg \cdot kg^{-1} \cdot hr^{-1}$, n = 6).

CHAPTER 4
RENOVASCULAR HYPERTENSION IN THE RAT: ROLES OF ANGIOTENSIN II, PROSTAGLANDINS, AND THROMBOXANE A_2

Introduction

The model of hypertension devised by Goldblatt in which renal artery constriction causes sustained hypertension is considered to be the result of the release of renin from the post-clip kidney (Lovenberg, 1987). A decrease in renal perfusion pressure, rather than renal ischemia, is the stimulus for this renin-dependent hypertension. This is a model for renovascular hypertension in patients with a unilateral constriction of one renal artery. Renovascular hypertension (RVH) in the rat has two phases: in the acute phase, both plasma renin activity (PRA) and BP are elevated whereas in the chronic phase, the BP remains elevated, but the PRA is variable and in some studies may decrease to normal (Pickering, 1990). Indeed, the model of hypertension most representative of human renovascular hypertension is the 2K,1C model of renal artery stenosis. As evidence for the role of renal artery stenosis in the etiology of renovascular hypertension, the hypertensive state is immediately resolved by removal of the clip, even in the chronic phase of the model (Brice et al., 1983).

The roles of the renin-angiotensin and PG systems in the etiology of RVH have been extensively studied but remain quite controversial. Angiotensin II can decrease RBF and release PGs; indeed, the renal vasoconstriction produced by an angiotensin II infusion is enhanced in some studies by indomethacin which prevents the generation of PGs. Thus, angiotensin II may release a vasodilator PG, such as PGI_2, which offsets its vasoconstrictor action (Aiken and Vane, 1973). On the other hand, PGI_2 is not a consistent renal vasodilator because of activation of renin release, and, in the rat, PGE_2 is a renal vasoconstrictor. Moreover, it is now recognized that indomethacin can cause vasoconstriction by cyclooxygenase-indepentent means. One study with the more specific cyclooxygenase inhibitor, aspirin, failed to confirm any increase in angiotensin II-induced renal vasoconstriction (Anderson et al., 1987). Finally, angiotensin II induces the release of clearly vasoconstrictor cyclooxygenase metabolites such as PGH_2 and TxA_2, and their production is also be blunted by indomethacin and aspirin. Indeed, indomethacin lowers the BP in rat models of renovascular hypertension, and aspirin lowers the BP in patients with renovascular hypertension. This may indicate that a PG vasoconstrictor may contribute to the hypertension (Stahl et al., 1981). On the other hand, these drugs blunt renin release and can reduce BP via this mechanism. One group claims that whereas a TxA_2 synthetase inhibitor does not affect the BP in angiotensin II-salt hypertensive rats, a TxA_2 receptor blocker lowers the BP

(Mistry and Nasjletti, 1988). Therefore, they implicate PGH_2, the precursor of TxA_2, as a mediator of the hypertensive response to angiotensin II. However, these authors have failed to produce any evidence that the TxA_2 synthetase inhibitor has reduced renal TxA_2 generation. In studies by Welch and Wilcox (1988b) and Welch et al.(1990), a prolonged period of pre-treatment with the TxA_2 synthetase inhibitor was required to reduce the level of basal and angiotensin II-stimulated renal TxB_2 excretion. When UK-38,485 is given in this way for 3 days before studying, it produces just as complete reduction of angiotensin II-induced pressor responses and renal vasoconstrictions as does a TxA_2/PGH_2 receptor antagonist. This suggests the alternative conclusion, that TxA_2, rather than PGH_2, is the predominant renal and systemic vasoconstrictor PG released by infusion of angiotensin II.

While the vascular reactivity to a TxA_2 mimetic increases in the early phase of RVH, the sensitivity to an α-adrenergic agonist increases later, and the reactivity to angiotensin II does not change (Zimmerman, 1987). Therefore, because of the increased reactivity to TxA_2 in the early phase, a role for PGs and TxA_2 is likely, especially at this stage of RVH. Recently, a TxA_2 antagonist has been shown to reduce the BP in the early phase of aortic coarctation-induced hypertension whereas indomethacin increased the BP (Lin et al., 1991). These findings further support the role of both vasodilatory and vasoconstrictive PGs in RVH but

leave unanswered the details of their mediation in the established model of human renovascular disease, the 2K,1C rat.

The locus of the renal effect of prostanoids in RVH has been indicated by studies of the clipped and unclipped kidneys. The clipped kidney of the 2K,1C model has increased glomerular PG and TxB_2 formation compared to the unclipped kidney in rats 6 weeks after clipping. Moreover, the GFR of the clipped kidneys decreases following treatment with indomethacin, while the GFR of the unclipped kidney is unaffected by indomethacin, and the MAP decreases with indomethacin (Stahl et al., 1984). These results suggest that vasodilator PGs may have a protective role in the clipped kidney while vasoconstrictor PGs may have a role in the production of the hypertension at some other location. Alternatively, they may indicate a nonspecific action of indomethacin independent of cyclooxygenase inhibition. Later studies in the 2K,1C rat showed that the GFR of the unclipped kidney is decreased and TxB_2 production is increased; treatment with a TxA_2 synthetase inhibitor and a TxA_2/PGH_2 receptor antagonist increased the GFR and reduced the systemic BP (Himmelstein and Klotman, 1989). Therefore, the vasoconstrictor effect of TxA_2 and its effect on the unclipped kidney may be implicated in the development of the hypertension in the 2K,1C model of RVH.

While some clarification of the location and roles of PGs and TxA_2 in the renal manifestations of RVH has resulted

from the previously cited studies, the temporal relationships between the effects of specific neurohumoral interactions on this form of hypertension have not been completely established. Specifically, while TxA_2 can mediate many of the short- and long-term effects of angiotensin II, it is unclear whether it mediates hypertension in the 2K,1C model only during the early angiotensin II-dependent phase. The aims of this study are to determine: (1) the role of TxA_2 in the early and late phases of hypertension in the 2K,1C model of RVH and (2) the interaction between the prostanoid and renin-angiotensin systems in control of hypertension during both phases of RVH in the 2K,1C model.

Methods

Experiments were performed on male Sprague-Dawley rats weighing 125-200 g. All rats were anesthetized with pentobarbital (60 $mg.kg^{-1}$ i.p.) for creation of a 2K,1C model of Goldblatt renovascular hypertension. The left kidney was exposed through a flank incision and a silver sheet clip (ID 0.22 mm) was placed around the left renal artery (2K,1C), or a sham operation was performed (sham). The wound was closed with steel clips, and the animals allowed to recover. The animals were maintained on standard rat chow (Rodent Laboratory Chow 5001, Ralston Purina Co., St. Louis, MO) with tap water to drink.

The animals were studied in two series with regard to the duration of their Goldblatt hypertension: the early phase of hypertension (E-2K,1C) was two to three weeks after clipping, and the late phase of hypertension (L-2K,1C) was two to three months after clipping. Sham-operated rats were studied at similar intervals. Within each series of E-2K,1C and L-2K,1C rats, groups were administered a vehicle, an angiotensin-converting enzyme inhibitor, an angiotensin receptor antagonist, a cyclooxygenase inhibitor, or a TxA_2/PGH_2 receptor antagonist.

On the experimental day, the animals were anesthetized with inactin (100 $mg \cdot kg^{-1}$; BYK Gulden Constanz, FRG). The MAP was recorded immediately after placement of a femoral artery catheter because this was the minimally stimulated condition of the rat. Rats were selected for further study at this time if their MAP exceeded 130 mmHg as calibrated on the electrical output from a pressure transducer (Model P 23, Gould, Oxnard, CA). A tracheostomy was performed and both jugular veins were cannulated; one venous catheter was used to transmit an infusion of 1% bovine serum albumin in 0.15 M NaCl delivered at 1 $ml \cdot hr^{-1}$ to maintain a euvolemic state whereas the other venous catheter was used to administer graded doses or infusions of the compounds to be studied. The bladder was catheterized for collection of urine from both kidneys and, in some cases, ureteral catheters were placed to collect urine from each kidney. The animals were

maintained in a normothermic state on a servo-controlled heated rat operating box.

After a 30 min equilibration period, a urine collection over a 30 min period in the basal state was obtained to assess the excretion of $6kPGF_{1\alpha}$ and TxB_2, and blood was sampled at the 30 min end-point for plasma renin activity (PRA). Thereafter, the animals received graded intravenous injections of an angiotensin-converting enzyme inhibitor (benazaprilat in 0.15 M NaCl, 0.01 to 100 $mg \cdot kg^{-1}$), a cyclooxygenase inhibitor (indomethacin in 1 M Na_2CO_3, 0.1 to 10 $mg \cdot kg^{-1}$), a TxA_2/PGH_2 receptor antagonist, (SQ-29,548 dissolved in ethanol and diluted in 0.15 M NaCl, 0.003 to 30 $mg \cdot kg^{-1}$), or a vehicle in 0.1 ml volumes at 30 min intervals over a total of 150 min. The MAP was recorded every 15 min. Additional groups of L-2K,1C animals received a 150 min infusion SQ-29,548 (8 $mg \cdot kg^{-1} \cdot hr^{-1}$), DuP753 (10 $mg \cdot kg^{-1} \cdot hr^{-1}$) (Jaiswal et al., 1991; Pieter et al., 1991), Captopril (300 $mg \cdot kg^{-1} \cdot hr^{-1}$), or a vehicle. These infusions were undertaken to test the effects of continuous maximal inhibition of angiotensin II subtype 1 receptor (AT-1) or TxA_2/PGH_2 receptor activation. Urine was analyzed for $6kPGF_{1\alpha}$ and TxB_2 and blood for PRA using previously described methods (see methods in chapter 3).

Statistical values were reported as the mean ± SEM. The data were analyzed by the Student's unpaired t-test. Values were taken as statistically significant at $p<0.05$.

Results

During the basal period, the MAP was increased by 29% for E-2K,1C compared to sham-operated rats ($p<0.005$); for L-2K,1C rats, the basal MAP was increased further by 48% ($p<0.005$) (Figure 4-1). In the basal period, the PRA was doubled for both the E-2K,1C and the L-2K,1C rats compared to shams ($p<0.005$) (Figure 4-2). Renal TxB_2 excretion from both kidneys was also doubled for both E-2K,1C and L-2K,1C rats ($p<0.05$) (Figure 4-3) (Table 4-1).

Within the series of E-2K,1C rats, the MAP was not altered by injections of vehicle. As anticipated, benazaprilat produced graded reductions of MAP to a level comparable to the MAP of shams with a maximal dose of angiotensin converting enzyme inhibitor (Figure 4-4). SQ-29,548 also caused dose-dependent reductions in MAP in the group of rats, but the reduction was only 50% as great as that produced by benazaprilat (Figure 4-4).

Within the L-2K,1C series, the MAP again was not altered by vehicle injections. However, in sharp contrast to E-2K,1C, the MAP was not reduced by any dose of benazaprilat (Figure 4-5). In contrast, SQ-29,548 produced graded depressor responses which lowered the MAP almost to the level of shams; a similar pattern was found with indomethacin (Figure 4-5).

Further information was obtained from the infusion protocols of the vehicle-infused rats in which the MAP was stable over 150 min. However, the infusion of SQ-29,548 caused a progressive fall in MAP to the level of shams by 90 min and remained at that level over the subsequently measured 60 min (Figure 4-6). In contrast, captopril was ineffective in reducing MAP or PRA, and DuP753 was quite without effect on MAP (data not shown).

In the L-2K,1C rats for which the urine was collected from the untouched kidneys, a four-fold increase in both $6kPGF_{1\alpha}$ and TxA_2 was demonstrated as compared to the values for shams ($p<0.005$) (Figure 4-7).

Discussion

The major findings of this study were that in the early phase of 2K,1C RVH, increased BP is dependent on both angiotensin II generation of cyclooxygenase products and activation of TxA_2/PGH_2 receptors. Hypertension was accompanied by increased PRA and TxA_2 excretion. In contrast, in the late phase, while the PRA and TxB_2 levels remained elevated, the hypertension was maintained by activation of TxA_2/PGH_2 receptors while losing its angiotensin II dependence.

In E-2K,1C rats, the hypertension is partially, but incompletely, reversed by a TxA_2/PGH_2 receptor antagonist. The early phase of RVH is angiotensin-dependent as

demonstrated by an increase in PRA and BP immediately after clipping the renal artery (Laragh et al., 1975), by a prompt reduction in PRA and BP following removal of the clip (Brice et al., 1983), and by a prompt lowering of BP by an angiotensin converting enzyme inhibitor or angiotensin II receptor antagonist (Masaki et al., 1977). The TxA_2 dependence of the early phase may be accorded to angiotensin-induced TxA_2 synthesis, since a TxA_2 synthetase inhibitor or a TxA_2/PGH_2 receptor antagonist can block two-thirds of the pressor response induced by angiotensin II and 80% of the renal vasoconstriction (Wilcox and Welch, 1990a; Wilcox and Welch, 1990b). Moreover, one study has shown the systemic hypertension of rats 4 week after placing a unilateral renal artery clip is reduced by a TxA_2 synthetase inhibitor or a TxA_2 receptor antagonist (Himmelstein and Klotman, 1989). In contrast, another study with a different model produced by complete ligation of the aorta between the renal arteries concluded that although hypertension was reduced by a TxA_2/PGH_2 receptor antagonist, it was resistant to a TxA_2 synthesis inhibitor (Lin et al., 1991). These authors assign PGH_2 the role of the major pressor prostanoid in their coarctation model. However, they have administered the TxA_2 synthetase inhibitor over a short-term which has been shown to be ineffective in reducing renal TxA_2 production as assessed from TxB_2 excretion by Welch and Wilcox (1989). It is possible that the TxA_2 synthetase inhibitor is unable to achieve an effective concentration in the kidney without

long-term administration. Therefore, our observation of a partial reduction in MAP with a TxA_2 receptor antagonist and a complete reversal of the hypertension with an angiotensin converting enzyme inhibitor in the early phase of 2K,1C hypertension was consistent with the findings of other investigators. As further evidence for the role of TxA_2 in angiotensin stimulated hypertension, an angiotensin II infusion increases the release of TxB_2 in the urine (Wilcox and Welch, 1990b),and the production of TxB_2 is increased from glomeruli from 6 week 2K,1C rats (Stahl et al., 1984) and from the contralateral kidneys of 4 week 2K,1C rats studied ex vivo during saline perfusion (Himmelstein and Klotman, 1989). Thus, blockade of the effects of TxA_2 release from the kidney and likely also from the blood vessels in response to angiotensin II may account for the antihypertensive properties of the TxA_2/PGH_2 receptor antagonist in this study in the E-2K,1C rat.

The classical model of Goldblatt hypertension produced by renal artery clipping is similar to human renovascular disease due to unilateral renal artery stenosis. The different model of renal hypertension mentioned above (Lin et al., 1991) uses complete aortic coartation between the renal arteries to produce a hypertensive, hyperreninemic state. The kidney distal to the ligation is very seriously ischemic and its perfusion is limited by collateral blood flow through lumbar or mesenteric vessels (Fernandes et al., 1976). While this model has some similarities to the renal artery stenosis

model, the dilation of the collateral circulation with relief of the renal ischemia may be the cause of the late phase reduction in renin rather than the progression of the pathophysiologic processes of renovascular hypertension. Certainly, in most patients with renovascular hypertension, the PRA remains mildly elevated following unilateral renal artery stenosis. This is mimicked by our model during the late phase of 2K,1C hypertension in the rat. Thus, the aortic coarctation model may not be equivalent to the renal artery stenosis model and may deficient as a model of the human disease.

In L-2K,1C rats, the MAP was resistant to an angiotensin converting enzyme inhibitor. This finding is contrasted with the effect in E-2K,1C rats in which the same angiotensin converting enzyme inhibitor restored BP to a normal level. The failure of PRA to normalize in the late phase is disturbing, since the elevated PRA predicts a good response to an angiotensin converting enzyme inhibitor. However, other investigators have reported that during the late phase of 2K,1C hypertension, rather than the fall in PRA as seen in the dog, the PRA in the rat may remain elevated in the chronic 2K,1C model (Watkins et al., 1976; Morton and Wallace, 1983). Moreover, our finding that a short-term administration of an angiotensin converting enzyme inhibitor did not reduce BP at this phase was in agreement with the results of others (Riegger et al., 1977). Riegger et al. (1977) find that very prolonged administration of sarslasin

reduced BP after 8-10 hr of infusion. However, other investigators have concluded that angiotensin II does not maintain the hypertension in the L-2K,1C (Masaki et al., 1977; Watkins et al., 1976). Indeed, others have failed to confirm this anti-hypertensive effect of long-term inhibition of angiotensin II receptors with saralasin or long-term inhibition of angiotensin II generation with captopril (Bing et al., 1981). In our study, we tested not only benazaprilat which is a direct-acting, non-sulpdidril angiotensin converting enzyme inhibitor and captopril which contains a sulphidril group, but also we used the novel an AT-1 angiotensin receptor antagonist, DuP753. None of these agents had any antihypertensive effect over 2.5 hr of infusion. These results demonstrate that the hypertension of this late phase of the 2K,1C model in the rat is independent of angiontensin II generation or AT-1 receptor activation. The possible role of AT-2 receptors was not investigated. Bolus administration of a TxA_2/PGH_2 receptor antagonist in the L-2K,1C rats caused a similar degree of antihypertensive response as seen in the E-2K,1C. Confirmation of this role of TxA_2 in L-2K,1C rats was revealed by the similar reduction in BP with graded doses of indomethacin. This observation suggested that the usual augmentation of BP with indomethacin in the presence of increased angiotensin II was no longer present because the response to TxA_2 and vasoconstrictor PGs overshadowed the response to vasodilator PGs. With prolonged administration to L-2K,1C rats by continuous infusion, the

TxA_2/PGH_2 receptor antagonist completely reversed the hypertension by 90 min. In contrast to our results, Lin et al. (1991) failed to detect an effect of a TxA_2/PGH_2 receptor antagonist during the late phase of aortic coarctation-induced hypertension when the PRA and TxB_2 excretion have returned to normal levels. Their disparate results provide further evidence that the 2K,1C model is not equivalent to the aortic coarctation model. In spite of the differences in PRA levels in our study, TxB_2 excretion in the L-2K,1C rats was maintained at the elevated levels present in E-2K,1C. This high renal TxB_2 excretion provided a rational mechanism for the finding of a reduction in hypertension with TxA_2/PGH_2 receptor blockade.

In the late phase, the unclipped kidney had an increased excretion of TxB_2 in our study. This finding is consistent with the increase in TxB_2 excreted from the unclipped kidney (Himmelstein and Klotman, 1989). However, others have found no increased excretion of PGs from a contralateral kidney from a 2K,1C model which is removed and perfused ex vivo.(Stahl et al., 1984). However, in our study, a concomittent increase of PGI_2 and TxA_2 was seen, while the ratio of PGI_2 to TxA_2 remained the same. The stimulus for the increased TxB_2 excretion from the contralateral kidney may be the increased renin release and intrarenal angiotensin II generation from the clipped kidney. This increased TxA_2 is in accord with the partial mediation of the effects of angiotensin II by TxA_2 (Wilcox, 1988b), whereas the increased

PGs may be a compensatory response to the vasoconstrictive effects of angiotensin II. Since our experiments were performed on the intact animal, the kidney might have responded to the vasoconstrictor effects of angiotensin II release from the clipped kidney; the isolated perfused kidney in the study of Himmelstein and Klotman, (1989) does not experience the vasoconstrictor effects of angiotensin II from this source. Thus, renal TxA_2 may play an important role in the pathophysiology of RVH within the contralateral kidney of 2K,1C rats which may not be fully evident in kidneys removed and perfused ex vivo.

Table 4-2 summarizes the responses in E-2K,1C and L-2K,1C renovascular rats and indicates that TxA_2/PGH_2 receptor blockade might have beneficial effects alone or in combination with an angiotensin synthesis inhibitor. Since elevated renin release, and consequent angiotensin II generation may maintain the glomerular capillary hydraulic pressure in the clipped kidney by efferent arteriolar vasoconstriction, total blockade of angiotensin II generation may have detrimental effects on the perfusion of the clipped kidney by reducing the efferent arteriolar resistance and reducing the transcapillary pressures that drive glomerular filtration (Hricik, 1990). It is possible that a moderation of the effects of this angiotensin II response on the efferent arteriole by TxA_2/PGH_2 receptor antagonism may have benefitial effects on the contralateral kidney without compromising the clipped kidney, since the concentration of

angiotensin II within the clipped kidney is very high and may be sufficient to maintain postglomerular resistance. However, this hypothesis remains to be investigated. In a model of prolonged 2K,1C hypertension, histological evidence of nephrosclerosis has been found in the contralateral kidney (Doyle and Duffy, 1980). Since increased TxA_2 production has been implicated in the renal hemodynamic and hypertensive changes in the contralateral kidney of 4 week 2K,1C rats (Himmelstein and Klotman, 1989), it is possible that a selective reduction in TxA_2/PGH_2 receptor activation may have benefitial effects in moderating some of the counterproductive structural adaptations in this form of hypertension.

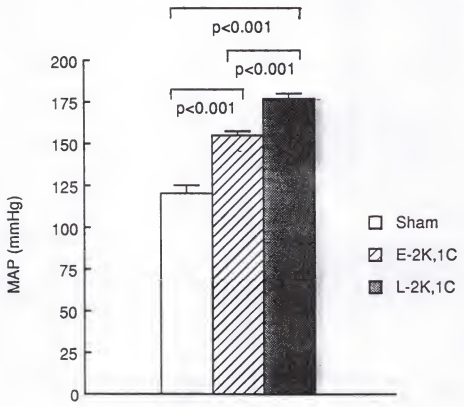

Figure 4-1. MAP following unilateral renal artery clip placement.

Mean ± SEM values for MAP in sham-operated control rats (n = 11), 2K,1C renovascular hypertensive rats studied 2-3 weeks after unilateral renal artery clipping (n = 43), or 2K,1C renovascular hypertensive rats studied 2-3 months after unilateral renal artery clipping (n = 48).

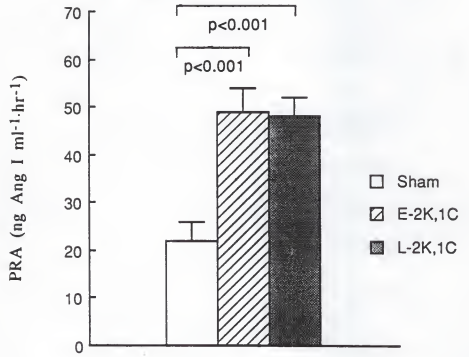

Figure 4-2. PRA following unilateral renal artery clip placement.

Mean ± SEM values for PRA in sham-operated control rats (n = 11), 2K,1C renovascular hypertensive rats studied 2-3 weeks after unilateral renal artery clipping (n = 43), or 2K,1C renovascular hypertensive rats studied 2-3 months after unilateral renal artery clipping (n = 48).

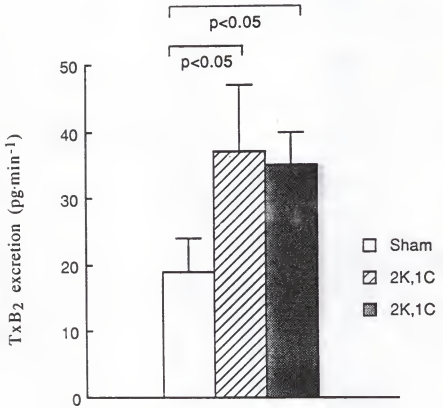

Figure 4-3. Total renal excretion of TxB_2.

Mean ± SEM values for excretion of TxB_2 in sham-operated control rats (n = 11), 2K,1C renovascular hypertensive rats studied 2-3 weeks after unilateral renal artery clipping (n = 43), or 2K,1C renovascular hypertensive rats studied 2-3 months after unilateral renal artery clipping (n = 48).

Table 4-1. The mean arterial blood pressure, plasma renin activity, and renal excretion of thromboxane B_2 by both kidneys of sham-operated control and early and late phase 2K,1C hypertensive rats.

	Sham	E-2K,1C	L-2K,1C
MAP (mmHg)	120±5 (n=11)	155±2 † (n=43)	177±3 † (n=48)
PRA (ng Ang I $ml^{-1} \cdot h^{-1}$)	22±4 (n=11)	49±5 † (n=36)	48±4 † (n=45)
TxB_2 excretion ($pg \cdot min^{-1}$)	19±5 (n=5)	37±10 * (n=11)	35±5 * (n=13)

Mean ± SEM values recorded under anesthesia in the basal period. E-2K,1C and L-2K,1C refer to rats with 2 kidneys, 1 clip hypertension studied 2-3 weeks and 2-3 months after clipping respectively. Compared to sham: *, $p<0.05$: †, $p<0.005$.

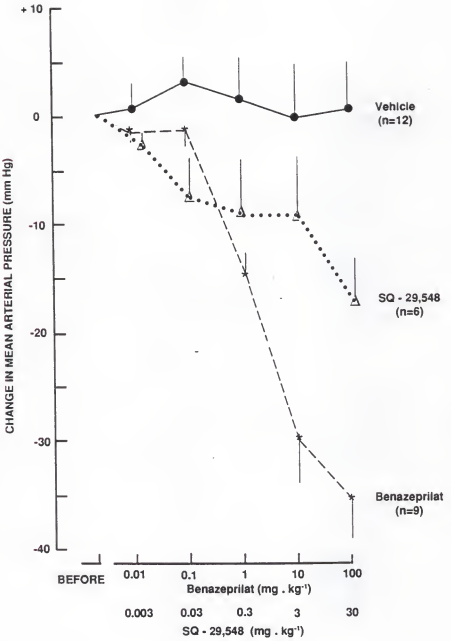

Figure 4-4. Effects of bolus injections of drugs on BP of 2K,1C rats (2-3 weeks).

Mean ± SEM values for change in MAP during cumulative dosages of vehicle (n = 12), benazaprilat (0.01-100 $mg \cdot kg^{-1}$, n = 9), or SQ-29,548 (0.003-30 $mg \cdot kg^{-1}$, n = 6) in 2K,1C renovascular hypertensive rats studied 2-3 weeks after unilateral renal artery clipping.

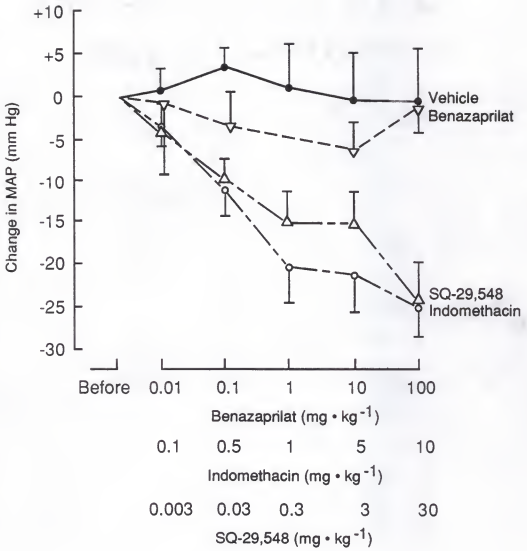

Figure 4-5. Effects of bolus injections of drugs on BP of 2K,1C rats (2-3 months).

Mean ± SEM values for change in MAP during cumulative dosages of vehicle (n = 11), benazaprilat (0.01-100 mg·kg^{-1}, n = 10), indomethacin (0.1-10 mg·kg^{-1}, n = 12), or SQ-29,548 (0.003-30 mg·kg^{-1}, n = 8) in 2K,1C renovascular hypertensive rats studied 2-3 months after unilateral renal artery clipping.

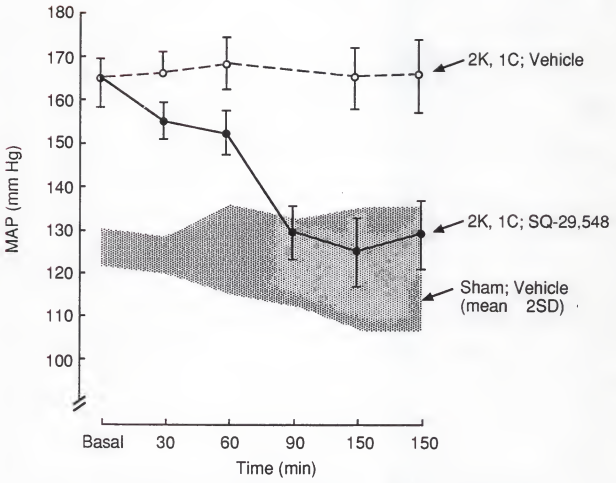

Figure 4-6. Effects of infusion of TxA_2/PGH_2 receptor antagonist on BP of 2K,1C rats (2-3 months).

Mean ± SEM values values for mean arterial pressure during a 150 min infusion of vehicle (n = 11), or SQ-29,548 (n = 8) in 2K,1C renovascular hypertensive rats studied 2-3 months after unilateral renal artery clipping. Mean ± 2 SD for MAP recorded in sham; vehicle (n = 3) studied 2-3 months after sham operations and receiving a vehicle infusion are shown for comparison.

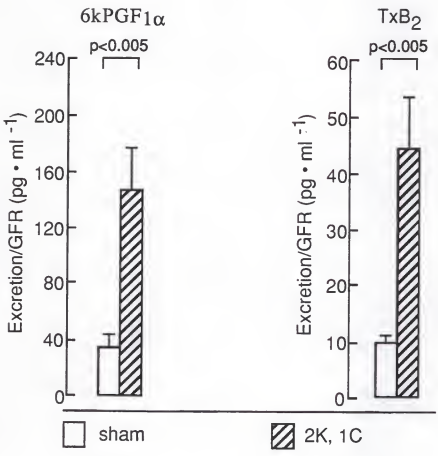

Figure 4-7. Renal excretion of prostacyclin and TxB_2 from unclipped kidneys of sham and 2K,1C rats.

Mean ± SEM values for excretion of $6kPGF_{1\alpha}$ and TxB_2 in unclipped kidneys of sham (n = 8) and 2K,1C (n = 8) renovascular hypertensive rats studied 2-3 months after unilateral renal artery clipping.

Table 4-2. Summary of results in 2K,1C renovascular hypertensive rats studied under anesthesia.

	Early phase (2-3 weeks)	Late phase (2-3 months)
Increase in BP	35 mmHg	57 mmHg
Increase in PRA	2-fold	2-fold
BP response to:		
ACEI	normalized	none
Indomethacin	reduced	reduced
TxA_2/PGH_2-RA	reduced	reduced or normalized

CHAPTER 5
THROMBOXANE A_2 RECEPTORS: SUBTYPE DIFFERENTIATION IN RAT GLOMERULI AND PLATELETS

Introduction

TxA_2 has been implicated in many models of hypertension and renal vasoconstriction. In the 2K,1C model of RVH, renal TxA_2 production is increased by the clipped and contralateral kidneys and appears to be a mediator of the hypertension and reduction in GFR (Himmelstein and Klotman, 1989; Stahl et al., 1984). Moreover, TxA_2 mediates 80-90% of the increase in RVS and the decrease in GFR caused by infusion of angiotensin II (Wilcox et al., 1990). In the remnant kidney and ureteral obstruction models, the kidney increases synthesis of TxA_2, and TxA_2 synthetase inhibition increases the GFR and RPF (Klotman et al., 1986; Purkerson et al., 1985).

TxA_2 is produced by isolated glomeruli (Folkert and Schlondorff, 1979) and cultured glomerular mesangial (Scharschmidt and Dunn, 1983) and epithelial (Petrulis et al., 1981) cells. TxA_2 or its stable mimetic, U-46,619, decreases GFR and RBF (Folger et al., 1989; Wilkes et al., 1989), contracts glomeruli and mesangial cells (Mené and Dunn, 1986), stimulates phosphoinositide turnover, and raises $[Ca^{2+}]_i$ in mesangial cells (Mené et al., 1988). The normal

physiological process of tubuloglomerular feedback is modulated by endogenous TxA_2 generation in the glomerulus (Welch and Wilcox, 1988). While these effects are evidence that TxA_2 has widespread and powerful actions in the kidney and glomerulus, only recently has a glomerular TxA_2 receptor been identified (Wilkes et al., 1989).

Whether TxA_2 receptors in platelets and in vascular tissues are different is somewhat controversial. Some pharmacologic (Swayne et al., 1988), biochemical (Hanasaki et al., 1989), and physiological (Akbar et al., 1985) studies have failed to demonstrate differences between TxA_2 receptors in platelets and in vascular tissues. However, use of a series of 13-azapinane TxA_2 analogues has demonstrated TxA_2/PGH_2 receptor subtypes by pharmacologic responses (Mais et al., 1985a, Mais et al., 1985b) or ligand binding studies (Masuda et al., 1991). Moreover, studies of 10,10-diflouro-TxA_2 stereoisomers in platelets and vascular tissues lend support to the notion that two subtypes of TxA_2 receptors exist (Morinelli et al., 1989b). Indeed, subsets of the vascular TxA_2 receptor have recently been proposed (Norman et al., 1990).

The present study is designed to characterize TxA_2/PGH_2 receptors in rat glomeruli and platelets. The iodinated TxA_2 agonist, [^{125}I]-BOP, is used as the ligand, and the rank order potency of a series of 13-azapinane TxA_2 antagonists to compete with [^{125}I]-BOP for binding is contrasted between glomerular membranes and platelets. Also rat glomerular

mesangial and epithelial cells are used to characterize the TxA_2/PGH_2 receptor in cells which constitute the glomerulus.

Methods

Preparation of Rat Whole Glomeruli

Rat glomeruli were prepared using modifications of Ballermann's method (Ballermann, 1988a; Ballermann et al., 1988b; Mizra, 1972). For each study, three 300-350 g Sprague-Dawley rats were pretreated with indomethacin (10 $mg \cdot kg^{-1}$ i.p.) 30 min prior to anesthesia to prevent endogenous TxA_2 synthesis. Rats were anesthetized with sodium pentobarbital (65 $mg \cdot kg^{-1}$ i.p.), their kidneys removed and placed in ice-cold phosphate-buffered saline (PBS) at pH 7.4, and the renal cortices dissected from the medulla. The tissue was minced with a razor blade and passed by gentle pressure through a stainless steel sieve having a pore size of 90 µm. The sieved glomeruli were washed with ice-cold PBS and passed through two further stainless steel sieves (180 and 150 µm) to remove large debris before being separated on a 75-µm sieve. This final separation step was repeated twice; a total of 500 ml of ice-cold PBS was used in the washing. The glomeruli were harvested into a 40-ml polycarbonate tube. This separation procedure yielded a preparation which was >95% pure glomeruli as assessed routinely by microscopic analysis. The glomeruli were

collected by centrifugation twice in 40 ml ice-cold PBS at 221 x *g* and 4°C for 2 min. Some experiments used this preparation of whole glomeruli.

Preparation of Rat Glomerular Membranes (RGM)

The RGM were prepared from whole glomeruli following cellular disruption by decanting them into iced hypotonic HEPES buffer (25 mM HEPES, 2 mM EDTA, 10 mM Mg^{++}, and 0.1 mM phenylmethylsulfonylfluoride) at pH 6.5 for 15 min. After centrifugation at 221 x *g* at 4°C for 2 min, the pellet was transfered to a 15-ml tube, resuspended in 5 ml of hypotonic HEPES buffer, and homogenized using six 10-second bursts (Polytron, Brinkman, Westbury, NY; setting 8) at 4°C. A 30-sec period intervened between bursts. The homogenate was sedimented at 221 x *g* at 4°C for 10 min to remove unbroken cells, nuclei, and debris. The resultant supernatant was centrifuged at 100,000 x *g* for 60 min at 4°C. The pellet was resuspended in 9 ml of ice-cold hypotonic HEPES buffer and homogenized with 10 strokes of a Dounce homogenizer (Wheaton, Millville, NJ). Protein concentration was determined using the Bio-rad (Richmond, CA) protein assay (Bradford, 1976). The remaining glomerular membranes were diluted in hypotonic HEPES buffer at pH 6.5 to a concentration of approximately 200 $\mu g \cdot ml^{-1}$ of protein and either frozen at -70°C or used immediately.

Preparation of Washed Rat Platelets

Sprague-Dawley rats were pretreated with indomethacin (10 mg·kg i.p.) to inhibit platelet TxA_2 generation. Twenty minutes thereafter, they were anesthetized with sodium pentobarbital (65 $mg \cdot kg^{-1}$ i.p.). Aortic blood was drawn into a syringe containing 10 μM indomethacin and 5 μM EDTA and was centrifuged at 100 x *g* for 20 min at room temperature, platelet-rich plasma was decanted and centrifuged at 1280 x *g* for 20 min at room temperature, and the pellet was resuspended in isotonic HEPES buffer (25 mM HEPES, 100 mM NaCl, 5 mM glucose, 2 mM EDTA, and 10 μM indomethacin), pH 7.4 to a final concentration of approximately 1 x 10^8 platelets·ml^{-1} for binding studies.

Binding of [^{125}I]-BOP

The incubation reaction (200 μl) consisted of hypotonic HEPES buffer (pH 6.5) and 20 μg of RGM at 30°C or isotonic HEPES buffer (pH 7.4) and 1 x 10^7 platelets at 37°C with ~ 0.01 nM (~3 to 5 x 10^4 cpm) of [^{125}I]-BOP in silanized glass tubes (12 x 75 mm) for 30 min. For equilibrium binding and competition binding experiments, concentrations of agonists or antagonists ranging from 10^{-11} to 10^{-4} M were added. The reaction was terminated by the addition of 4 ml of ice-cold hypotonic HEPES buffer at pH 6.5 for RGM or isotonic HEPES buffer at pH 7.4 for WRP, followed by rapid filtration

through Whatman GF/C glass fiber filters (Whatman, Inc., Clifton, NJ). The filters were washed 3 times with 4 ml of ice-cold buffer. The entire filtration procedure was complete within 10 sec. Nonspecific binding was defined as that amount of radioactivity bound in the presence of a maximum concentration of a TXA_2/PGH_2 receptor antagonist (10 μM L-657,925) (Mais et al., 1989).

Experiments for determining the association rate were carried out by incubating the membranes for varied time periods at 37°C with [^{125}I]-BOP (~5 x 10^4 cpm plus 0.01 nM [^{127}I]-BOP). Termination of the incubation was carried out as described above. For dissociation experiments, glomerular membranes were incubated with [^{125}I]-BOP as described above for 30 min at 37°C. The radioligand was displaced with L-657,925 (10 μM) and the dissociation terminated at specific time points by the addition of ice-cold hypotonic HEPES buffer, as described above.

Measurement of Inositol Phosphate Release from Glomeruli

Inositol phosphate release was measured using a modification of the method described by Gonzales and Crews (1984). Isolated whole glomeruli from four rat kidneys prepared as described above were prelabelled by incubating the glomeruli with 40-50 μCi of [^{3}H]-myo-inositol in 0.75-1.0 ml of Krebs-Ringers phosphate buffer (KRB) (118 mM NaCl, 24.8 mM $NaHCO_3$, 4.7 mM KCl, 0.75 mM $CaCl_2$, 1.18 mM KH_2PO_4, 1.18 mM

$MgSO_4$, 10 mM glucose) at pH 7.4 bubbled with 95:5% O_2:CO_2 at 37°C for 1 h. The excess label was removed by washing with 2 ml KRB four times, and the glomeruli were resuspended in KRB. Aliquots of 50 µl of the glomerular suspension were transfered to 12 x 75 mm Sarstedt tubes (Sarstedt, Inc., Newton, NC) and incubated in Li-KRB (108 mM NaCl, 24.8 mM $NaHCO_3$, 4.7 mM KCl, 0.75 mM $CaCl_2$, 1.18 mM KH_2PO_4, 1.18 mM $MgSO_4$, 10 mM glucose, 10 mM LiCl) and 1 µM myo-inositol with various agonist concentrations for 1 h. Incubation was terminated by adding 1 ml of ice-cold chloroform/methanol (1:2) to each tube. For extraction of lipids, water (0.35 ml) and chloroform (0.35 ml) were added, and the mixture was shaken for 10 min and centrifuged for 5 min at 883 x *g*. Inositol phosphates were separated by transferring 0.75 ml of the aqueous phase onto a 1 ml slurry of H_2O Dowex anion-exchange resin (AG 1X8, 100-200 mesh, Bio-Rad, Richmond, CA) in formate form in a column and eluting with 5 ml 1 M formate buffer. The lipid phase was transferred to scintillation vials in 200 µl aliquots and dried, and scintillation fluid was added for counting of both aqueous and lipid phases. Total [^{3}H]-inositol phosphate release was expressed fractional release. IP fractional release (FR) is calculated as the ratio of total inositol phosphates released to total myo-inositol (Ins) incorporated into membrane phospholipids using the following formula (Gonzales and Crews, 1984): FR =

DPM [^{3}H]-IP in aqueous phase

DPM [^{3}H]-IP in aqueous phase + DPM [^{3}H]-Ins in organic phase

Culture of Rat Mesangial Cells.

Mesangial cells were harvested from male Sprague-Dawley rats weighing 150-200 g following previously published techniques (Mené and Dunn, 1986). Eight kidneys from 4 rats were used to set up each primary culture using standard aseptic techniques. The rats were anesthetized with pentobarbitol (65 mg·kg^{-1} i.p.). Their kidneys were removed and transferred to a laminar flow hood where the kidneys were decapsulated and transected. The cortices were separated, and the glomeruli were harvested by mechanical sieving. The culture medium was RPMI 1640 containing 16% fetal bovine serum, and the cells were grown at 37°C in a 5% CO_2 incubator. After 3 to 4 weeks the mesangial cells had overgrown the other cells, become confluent, and constituted greater than 90% of the cells in the culture. After one or two passages, the cultures consisted exclusively of mesangial cells. The cells were then subcultured every 7 to 10 days using mild trypsinization. Mesangial cells in our culture preparations were routinely identified by 1) spindle or stellate shape under phase electron microscopy, 2) presence of microfilament bundles under transmission electron microscopy, 3) ability to proliferate in medium containing

d-valine which condition inhibits fibroblast growth, 4) resistance to the effects of the glomerular epithelial cytotoxin--aminonucleoside of puromycin (100 $\mu g \cdot ml^{-1}$), 5) cell detachment and lysis after overnight exposure to the mesangial cytotoxin--mitomycin (10 $\mu g \cdot ml^{-1}$), 6) presence of immunofluorescence staining for anti-smooth muscle specific myosin antibodies, 7) absence of immunofluorescence staining for anti-factor VIII antibodies which excluded the presence of endothelial cells, and 8) contractile response to angiotensin II.

Preparation of Intact Rat Mesangial Cells and Membranes

Mesangial cell cultures were grown in 100-mm diameter culture dishes. The media was removed, and the cells were washed twice with ice-cold KRB at pH 7.4. The cells were scraped off the dishes with a disposable cell scraper (Fisher Scientific, Pittsburg, PA) and centrifuged at 1,000 rpm for 5 min. The pellet containing intact cells was resuspended in KRB for binding studies or preparation of cell membranes. For the later procedure, the cells were homogenized with a tissumizer (Tekmar Co., Cincinnati, OH; setting 80) for 10 sec times 4. The homogenate was centrifuged a 600 x *g* for 10 min at 4°C. The resultant supernatant was aspirated and centrifuged at 20,000 *g* for 30 min at 4°C. The pellet was resuspended in KRB and centrifuged again at 20,000 *g* for 30

min. The final pellet was resuspended in 1 ml KRB for binding studies.

For other binding studies, mesangial cells were grown on 24-well plates and binding was performed directly on the plates. Similar methods were used for binding studies on rat glomerular epithelial cells. In addition to binding with I-BOP, other studies were performed using $[^3H]$-$PGF_{2\alpha}$.

Measurement of Inositol Phosphate Release from Mesangial Cells

Mesangial cells were plated at a density of 1×10^4 cells cm^{-2} in 6-well plates and allowed to grow to confluency for 7-14 days. The media (RPMI 1640 + FCS 16%) was changed every 3-4 days. The cells were incubated with myo-$[^3H]$-inositol at a concentration of 2 $\mu Ci \cdot ml^{-1}$ in 1.5 ml MEM resting media (MEM + FCS 0.5% + BSA 0.9%) for 48 hours. Cells were used between passages 2 to 8. Stimulation experiments were performed in triplicate with a zero-control (vehicle), a 15-min-control (vehicle), agonist, and agonist + antagonist. The radiolabelling media was removed and replaced with Li-KRB for the 3 zero-control wells (1000 $\mu l \cdot well^{-1}$); this media was removed and transferred to a test tubes; and 1000 µl of ice-cold methanol was immediately added to the wells to terminate the incubations. The cells were scraped from the bottom of the wells using a rubber policeman and transferred to the test tubes; the wells were washed with 500 ml of methanol which was transferred to the tubes for a

total volume of 2500 µl·tube^{-1} in the aqueous phase. The 15-min-control wells were incubated in 1000 µl Li-KRB, agonist wells in 900 µl Li-KRB + 100 µl agonist (10X), and antagonist wells in 890 µl Li-KRB + 100 µl agonist (10X) + 10 µl antagonist (10X) for 15 minutes. The reactions were stopped as described above. Chloroform (1000 µl) was added to each tube, and they were agitated x 10 min and centrifuged at 2000 rpm x 5 min to extract the lipid phase. Fresh, well-stirred Dowex anion exchange resin (AG 1x8, 100-200 mesh, Bio-Rad, Richmond, CA) in formate form (1 ml) was added to Poly-Prep chromatography columns (10-ml, Bio-Rad, Richmond, CA). To each column, 750 µl of aqueous (top) phase was added, allowed to drain completely, and washed with 7 ml of distilled water x 2 to remove free myo-[^{3}H]-inositol. Total [^{3}H]-inositol phosphates in the aqueous phase were eluted into scintillation vials with 5 ml formate buffer (1 M), and 18 ml ScintiVerse L (Fisher Scientific, Pittsburg, PA) was added for counting in a Beckman LS 7000 scintilation counter. The remaining aqueous phase and the protein interphase were aspirated. Total [^{3}H]-inositol phosphates (200 µl) in the organic (bottom) phase were transferred to scintillation vials, evaporated with nitrogen, and 8 ml of ScintiLene (Fisher Scientific, Pittsburg,PA) added for counting.

Materials

The synthesis of [^{127}I]-BOP and [^{125}I]-BOP was carried out as described previously by Morinelli et al. (1989a). The U-46,619 was a gift from Upjohn Company (Kalamazoo, MI). The SQ-29,548 was a gift from Squibb Institute for Medical Research (Princeton, NJ). The 13-azapinane compounds were synthesized as described previously (Mais et al., 1985a). Sodium pentobarbital was obtained from Butler Co. (Columbus, OH). All other reagents were obtained from Sigma Chemical Co. (St. Louis, MO).

Statistics

Data from equilibrium binding experiments were analyzed using the LIGAND (Munson and Rodbard, 1980) computer program and data from competitive binding experiments were analyzed using EBDA (McPherson, 1983). The K_d and IC_{50} values were represented as the mean ± S.E.M. The IC_{50} was defined as the concentration of competing agent that produced a 50% inhibition of specifically bound [^{125}I]-BOP. The EC_{50} was defined as the agonist concentration producing a half-maximal response. The IC_{50} and EC_{50} values were determined from a log-logit transformation of the individual concentration-response curves. Spearman's rank order correlation was used to determine differences between the rank orders of binding in the washed rat platelets and RGM.

Results

Binding of [^{125}I]-BOP to Glomerular Membranes

Binding conditions were optimized for pH, tissue concentration, Mg^{++}, Ca^{++}, and radioligand concentration. The effect of pH on the specific binding to whole rat glomeruli was studied over the pH range 6.0 to 8.0 and was maximal at pH 6.5 (Figure 5-1). Binding was linear over a tissue concentration of 4 to 33 μg·ml, Mg^{++} concentration was optimal at 5 to 10 mM, whereas Ca^{++} and Na^{+} had no effect on binding (data not shown). Specific binding was 75 ± 2 % (n = 14) of total binding.

The association rate constant (k_1) for the ligand-binding site complex was determined from the time course for binding of [^{125}I]-BOP. Binding approached equilibrium within 12 min at 37°C and 15 min at 30°C. The observed rate constant (k_{obs}) was given by the slope of the transformed association curve and was found to be 0.45 min^{-1}. Dissociation of the receptor-ligand complex demonstrated an exponential decrease in binding. Linear transformation of these data gave a straight line with a negative slope, which is a measure of the dissociation rate constant (k_{-1}). The $t_{1/2}$ at 37°C was 7 min and at 30°C was 21 min. The value for k_{-1} was found to be 0.1 min^{-1}. The association rate constant, k_1, was determined from the formula: $k_1 = (k_{obs}-k_{-1})\cdot[L]^{-1}$,

where [L] = the total ligand concentration. The calculated k_1 was determined to be 7.78 x 10^8 $M^{-1} \cdot min^{-1}$. The kinetically determined dissociation constant, K_d, was determined from the formula: $K_d = k_{-1} \cdot k_1^{-1}$. At 37°C the K_d was determined to be 128 pM and 134 pM for two separate experiments.

The binding of [^{125}I]-BOP to RGM measured at equilibrium was saturable. Scatchard analysis of the binding isotherms by nonlinear least squares curve-fitting revealed a K_d of 318 ± 55 pM and a maximum binding capacity (B_{max}) of 260 ± 62 $fmol \cdot mg^{-1}$ protein (n = 14) (Figure 5-2).

Competition Binding Studies in RGM and Washed Rat Platelets

To establish that [^{125}I]-BOP was interacting with specific TxA_2/PGH_2 receptors on the RGM, competition binding experiments were performed using the TxA_2/PGH_2 receptor agonist U-46,619, and the TxA_2/PGH_2 receptor antagonist SQ-29,548, and the stereoisomeric pair of antagonists, L-657,925 and L-657,926 (Figures 5-3a and 5-3b). The rank order for these compounds was L-657,925 > U-46,619 > SQ-29,548 > L-657,926. Six 13-azapinane TxA_2 antagonists were used to displace [^{125}I]-BOP in order to establish a rank order of potency for binding of RGM and washed rat platelets. These experiments were performed at pH 7.4 because that was the optimum binding pH for 13-azapinane TxA_2 analogues (Mais et al., 1985a). The rank order in RGM was I-PTA-OH > ONO11120(2) > ONO11120(1) > mI-PTA-PO > PTA-TPO > PTA-NO; the

rank order in washed rat platelets was PTA-TPO > I-PTA-OH > mI-PTA-PO > PTA-NO > ONO11120(2) > ONO11120(1). There was no significant correlation between the rank order of potencies of the K_d values for the 13-azapinane TxA_2 analogues in displacement of I-BOP in RGM and washed rat platelets ($p = 0.872$) (Figure 5-4). All six 13-azapinane TxA_2 compounds were more potent in washed rat platelets than in RGM. A TxA_2/PGH_2 receptor antagonist, [^{125}I]-PTA-OH, failed to specifically bind to RGM (data not shown).

Phosphoinositide Hydrolysis from Glomeruli

Stimulation of the TxA_2/PGH_2 receptor in other tissues and mesangial cells has been associated with activation of phospholipase C (Mené et al., 1988; Halushka et al., 1989). The TxA_2/PGH_2 agonist I-BOP stimulated inositol phosphate release in rat whole glomeruli. A concentration-response relationship was elicited with an EC_{50} of 1.99 ± 0.43 nM, n = 4 (Figure 5-5). The antagonist L-657,925, at a concentration of 10 μM, was able to completely block the maximal stimulation with I-BOP at a concentration of 1 μM. The stereoisomers L-657,925 and L-657,926 had IC_{50} values of 27.4 ± 5.5 nM (n = 4) and 811 ± 116 nM (n = 3) for antagonizing I-BOP (2 nM) stimulated inositol phosphate production.

Binding to [^{125}I]-BOP and [^{3}H]-$PGF_{2\alpha}$ to Glomerular Mesangial and Epithelial Cells

Preliminary binding studies on cultured intact mesangial cells with [^{125}I]-BOP revealed 19% specific binding whereas binding to cultured mesangial cell membranes had 14% specific binding (Table 5-2). No specific binding was identified for cultured mesangial cells with [^{3}H]-$PGF_{2\alpha}$ nor was any found with either ligand for cultured epithelial cells (data not shown).

Phosphoinositide Hydrolysis from Mesangial Cells

I-BOP stimulated cultured mesangial cells in a dose-dependent manner with an EC_{50} of approximately 500 nM (Figure 5-6). No stimulation above control was evident at 10 nM and stimulation was complete at 1 μM concentration of I-BOP. The TxA_2/PGH_2 receptor antagonists L-657,925 (10 μM), SQ-29,548 (1 μM), and mI-PTA-PO (5 μM) were unable to antagonize the stimulation of mesangial cells with I-BOP (1 μM) (Figure 5-7).

Discussion

This study describes the binding of [^{125}I]-BOP to TxA_2/PGH_2 receptors in rat glomerular membranes, its competition with a series of receptor antagonists in RGM and washed platelets, and its stimulation of inositol phosphate release in rat whole glomeruli. Previous reports have shown that [^{125}I]-BOP binds to washed human platelets with K_d values

of 234 pM and 2.2 nM (Dorn, 1989; Morinelli et al., 1989a), to human vascular smooth muscle cells with a K_d of 310 pM (Morinelli et al., 1990), and to washed rat platelets with a K_d of 205 pM (Masuda et al., 1991). In this study the K_d for I-BOP in RGM was 318 pM. Thus, the K_d value is similar to that reported for the high affinity binding site in WHP and washed rat platelets. The only other report of a binding site for a radiolabelled TxA_2 analogue in glomeruli used [^{3}H]-SQ-29,548 and whole glomeruli (Wilkes et al., 1989). The K_d (14 nM) reported in that study for SQ-29,548 is lower than that found in the present study (39 nM) (Cheng and Prusoff equation was used to convert the IC_{50} value of 41 to a K_d value of 39). The reason for this discrepancy may be the fact that the pH optimum for SQ-29,548 is 7.4 and for I-BOP is 6.5 (Mayeux et al., 1991). Since the competition binding assays for SQ-29,548 were performed at pH 6.5 in the current study, this is the most likely explanation for the higher K_d reported in this study. The B_{max} for SQ-29,548 is 361 $fmol \cdot mg^{-1}$ protein in the whole glomeruli, whereas in glomerular membranes I-BOP had a B_{max} of 260 $fmol \cdot mg^{-1}$ protein. The differences in B_{max} values may be accounted for by a combination of the low specific binding for SQ-29,548 (42-82%) and the use of whole glomeruli compared to the high specific binding for I-BOP and the use of glomerular membranes.

The competition binding studies with analogues of TxA_2 indicate that I-BOP binds to the TxA_2/PGH_2 receptor in RGM in

a specific manner. To unequivocally establish specific binding to the TxA_2 receptor in RGM, competition studies were performed with the stereoisomers L-657,925 (-) and L-657,926 (+). L-657,925 was similar in potency to I-BOP and was 460 times more potent than L-657,926 in competition binding studies. The difference in potencies for the two compounds strongly supports the notion that I-BOP is binding to a specific TxA_2 receptor.

The possibility that subtypes of the TxA_2/PGH_2 receptor exist in washed rat platelets and RGM was determined by comparing the rank order of potencies of six 13-azapinane TxA_2 analogues in competition binding assays in these two tissues. The six members of the homologous series had K_d values which were not significantly different from each other in platelets. The K_d values for the compounds in RGM were higher than for those in platelets and were significantly different within the series. Spearman's rank order test demonstrated that there is no reasonable probability of a correlation existing between the rank orders of binding in RGM and washed rat platelets. A difference in the rank orders of binding for these compounds has previously been noted between washed rat platelets and cultured rat aortic smooth muscle cells (RASMC) using [^{125}I]-BOP (Masuda et al., 1991). These results suggest that different subtypes of the TxA_2/PGH_2 receptor exist in RGM and washed rat platelets, and that the subtypes can be distinguished by compounds with

different substitutions on the terminal end of the ω-side chain of the 13-azapinane TxA_2 analogues.

Previous studies with the two epimers of ONO11120 indicate that they may identify different receptor subtypes in RGM and washed rat platelets. Mais et al. (1985b) has shown that the relative potency of ONO11120(1) compared to ONO11120(2) (IC_{50} values: 90 nM / 30 nM = 3) in blocking saphenous vein contractions differ from that in WHP aggregation (IC_{50} values: 550 nM / 460 nM = 1.2). Masuda et al. (1991) has demonstrated significant differences in the binding of these two epimers in RASMC (IC_{50} values: 596 nM / 313 nM = 1.9) but not in washed rat platelets (IC_{50} values: 142 nM / 124 nM = 1.15) or WHP (IC_{50} values: 383 nM / 370 nM = 1.04). This study did not show any significant difference in the K_d values for binding of the ONO11120 epimers in RGM (IC_{50} values: 1106 nM / 607 nM = 1.82) nor in washed rat platelets (IC_{50} values: 458 nM / 343 nM = 1.33). Collectively, these studies suggest, but do not prove, that the TxA_2 receptor in RGM may be different than that in vascular smooth muscle and that the orientation of the C-15 hydroxyl group may influence the potency.

Previous studies have not related TxA_2 receptor binding to a response in whole glomeruli. Changes in renal clearance (Folger et al., 1989; Wilkes et al., 1989) or PG and TxA_2 release (Hassid et al., 1979; Sraer et al., 1982) are too far removed from receptor binding and may be modified by compensatory reflexes and hormone release which preclude

making a correlation with receptor binding. Indeed, renal hemodynamic responses to U-46,619 involve predominately or exclusively renal vascular smooth muscle whose receptors may differ from those on glomeruli. Thus, the glomerular capillaries contribute little to total RVR; the only determinant of glomerular filtration which is truly intraglomerular is the ultrafiltration coefficient which is reported not to change consistently during infusion of U-46,619 (Baylis, 1987). Even the RVR changes during infusion of U-46,619 may be an indirect consequence of an enhanced tubular transport with secondary activation of tubuloglomerular feedback and afferent arteriolar vasoconstriction (Welch and Wilcox, 1990). Therefore, to gauge the functional relevence of the results of the glomerular binding studies, we used a biochemical response involving isolated whole glomeruli. One advantage of selecting an agonist for the receptor binding studies is that it can also be used to test the biochemical response in the same tissue. We have elected to study the receptor-mediated increase in phosphoinositide hydrolysis in whole rat glomeruli. Phosphoinositide turnover is enhanced by angiotensin II and bradykinin in isolated rat glomeruli (Ochi et al., 1987, Sekar et al., 1990) and by TxA_2 mimetics in whole glomeruli, platelets, and vascular smooth muscle cells. I-BOP stimulated phosphoinositide turnover in a concentration-dependent manner which was blocked stereoselectively by L-657,925 and L-657,926. The relative

potencies of these two stereoisomers in whole glomeruli (EC_{50} values: 811 nM / 27 nM = 30) were comparable to that reported for washed human platelet aggregation (K_d values: 186 nM / 1.8 nM = 103) (Mais et al., 1989). These phosphoinositide turnover results indicate that I-BOP is stimulating a functional receptor in rat glomeruli. Whether this TxA_2/PGH_2 receptor evokes a physiologic response in the glomerulus remains unresolved. However, the ratio for the potency of these compounds in the binding assay (460) was greater than that found in the phosphoinositide turnover study. The reason for the discrepancy is uncertain but may relate to differences between membranes and whole glomeruli.

The glomerular mesangial cell has been espoused to alter the glomerular ultrafiltration coefficient by contracting and decreasing the glomerular surface area by a phosphoinositide hydrolysis pathway. The TxA_2 mimetic U-46,619 has been shown to stimulate phosphoinositide hydrolysis with maximal stimulation at 10 μM concentration (Mené et al., 1989b). In our studies, I-BOP stimulated phosphoinositide hydrolysis of mesangial cells in a dose-dependent manner with a EC_{50} value of approximately 500 nM. I-BOP was about ten times more potent than U-46,619. However, three TxA_2/PGH_2 receptor antagonists were entirely ineffective in antagonizing the I-BOP stimulated phosphoinositide hydrolysis. The high EC_{50} and the inability to antagonize indicate that I-BOP stimulation of phosphoinositide hydrolysis in mesangial cells is receptor-independent. Indeed, Mené et al. (1989a) report

antagonism of $[Ca^{2+}]_i$ release but not phosphoinositide hydrolysis stimulated by U-46,619. As further evidence for the lack of a specific receptor-mediated stimulation of phosphoinositide hydrolysis in mesangial cells, we demonstrated very little specific binding of $[^{125}I]$-BOP to mesangial cells.

Since Mené et al., 1988 have suggested that U-46,619 may be binding to the $PGF_{2\alpha}$ receptor, we performed binding studies with $[^{3}H]$-$PGF_{2\alpha}$ on mesangial cells. No specific binding was demonstrated for $[^{3}H]$-$PGF_{2\alpha}$. Thus, we conclude that I-BOP cannot be binding nonselectively to $PGF_{2\alpha}$ receptors in mesangial cells.

Since I-BOP was not binding specifically to mesangial cells to stimulate phosphoinositide hydrolysis as it did in glomeruli, we attempted to identify TxA_2/PGH_2 receptors on the epithelial cells of the glomerulus. However, no specific binding of $[^{125}I]$-BOP was present on these cells. Therefore, the most likely location of the TxA_2/PGH_2 receptor in the glomerulus is the endothelial cell; further studies involving cultured glomerular endothelial cells will be needed to positively establish the cell linked to phosphoinositide hydrolysis in the glomerulus.

In conclusion, I-BOP recognizes specific and functional TxA_2/PGH_2 receptors in RGM, and competitive binding assays support the existence of subtypes of the TxA_2/PGH_2 receptor in different tissues from the same species. The 13-azapinane TxA_2 analogues appear to be unique in their ability to

discriminate these receptor subtypes in several tissues compared to a heterologous series of TxA_2 analogues. Since the glomerulus is a heterogeneous mixture of three major cell types, the determination of which cell types possess the TxA_2/PGH_2 receptors will only be resolved with further studies on cultures of glomerular mesangial, epithelial, and endothelial cells.

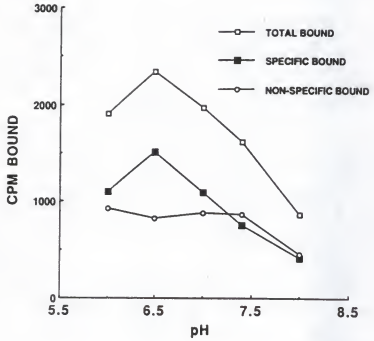

Figure 5-1. Effect of pH on total, specific, and nonspecific binding of [^{125}I]-BOP.

Nonspecific binding was defined as the amount of [^{125}I]-BOP bound in the presence of 10 μM L-657,925. Each point represents the mean of four experiments with duplicate determinations.

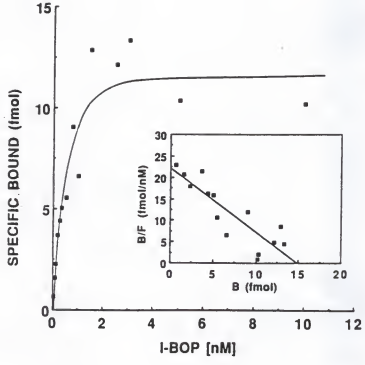

Figure 5-2. Representative equilibrium binding analysis of [^{125}I]-BOP to rat glomerular membranes.

Inset shows Scatchard analysis of saturation binding data. Bound/free (B/F) (fmol/nM) is shown as a function of specifically bound (B) (fmol). The K_d was 497 pM with B_{max} = 869 fmol/mg protein. These data are representative of 14 experiments.

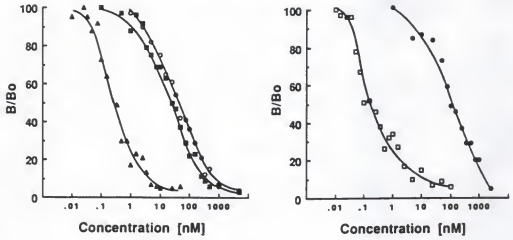

Figure 5-3. Competition binding curves with [^{125}I]-BOP in rat glomerular membranes.

Values are represented as the mean ± SEM for the indicated number of experiments. B = bound cpm at a given concentration. B_o = bound cpm for [^{125}I]-BOP and no added [^{127}I]-BOP. Panel on left: IC_{50} = 0.34 ± 0.05 nM for [^{127}I]-BOP, n = 4; 22 ± 6 nM for U-46,619, n = 3; 41 ± 7 nM for SQ-29,548, n = 4; panel on right: IC_{50}.= 27 ± 0.04 nM for L-657,925, n = 3; 124 ± 0 nM for L-657,925, n = 2.

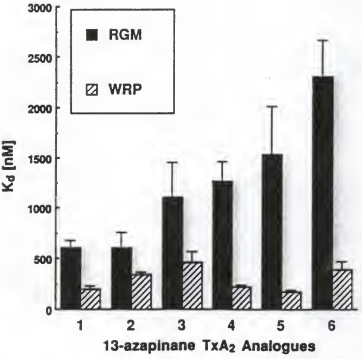

Figure 5-4. Comparison of rank order potencies for competition binding of 13-azapinane TxA_2 analogues in rat glomerular membranes (RGM) and washed platelets (WRP).

Mean ± SEM.values for K_d values in nM. Spearman's rank order test was used for comparing the rank orders. P = 0.872, n = 2-7. Compounds shown in numbers are as follows: 1, I-PTA-OH; 2, ONO11120(2); 3, ONO11120(1); 4, mI-PTA-PO; 5, PTA-TPO; 6, PTA-NO (see ref Mais et al., 1985a for structures).

Table 5-1. The K_d values for displacement of [^{125}I]-BOP by 13-azapinane TxA_2 analogues: comparison between rat glomerular membranes and washed rat platelets.

Antagonist	K_d values (nM) RGM	WRP
I-PTA-OH	601 ± 75 (2)	201 ± 27 (3)
ONO11120 (epimer 2)	607 ± 145 (6)	343 ± 23 (4)
ONO11120 (epimer 1)	1106 ± 345 (6)	458 ± 104 (5)
mI-PTA-OH	1262 ± 199 (7)	222 ± 19 (3)
PTA-TPO	1530 ± 475 (4)	178 ± 16 (4)
PTA-NO	2301 ± 358 (6)	387 ± 80 (4)

Mean ± SEM values for K_d of 13-azapinane TxA_2 analogues for competing with [^{125}I]-BOP binding to RGM and WHP. All K_d values are nM concentrations. Parentheses contain the number of experiments.

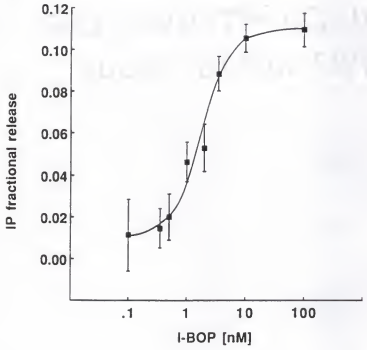

Figure 5-5. Fractional release of inositol phosphates (IP) from rat whole glomeruli.

Mean ± SEM values for dose-dependent increases in inositol phosphates were induced by various concentrations of [^{127}I]-BOP. The EC_{50} value was 1.99 ± 0.43 nM, n = 4.

Table 5-2. Comparison of I-BOP binding to platelets, glomeruli, and mesasangial cells.

Preparation	Specific binding (%)
Human platelets	92
Rat platelets	89
Rat whole glomeruli	57
Cultured intact mesangial cells	19
Membranes of mesangial cells	14

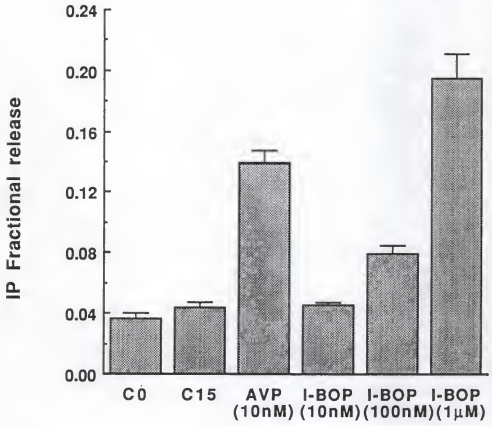

Figure 5-6. Fractional release of inositol phosphates from mesangial cells stimulated with a AVP and a TxA_2 mimetic.

Mean ± SEM values for triplicate measurements of inositol phosphate release during stimulation with vehicle (control) at 0 and 15 min, AVP (10 nM), and I-BOP (10 nM, 100 nM, and 1 μM).

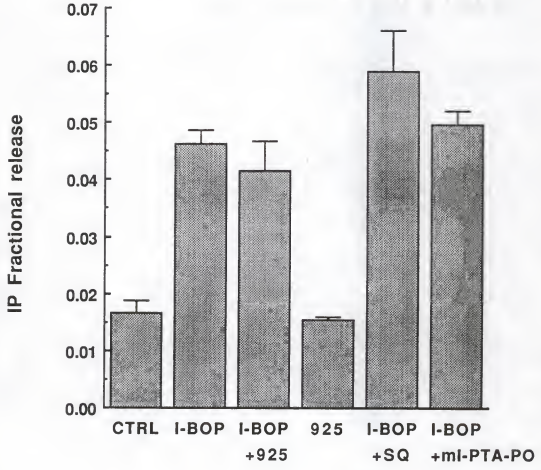

Figure 5-7. Fractional release of inositol phosphates from mesangial cells stimulated with a TxA_2 mimetic and with TxA_2/PGH_2 receptor antagonists.

Mean ± SEM values for triplicate measurements of inositol phosphate release during stimulation with vehicle (control), I-BOP (1 μM), I-BOP (1 μM) + L-657,925 (925, 10 μM), L-657,925 (925, 10 μM),.I-BOP (1 μM) + SQ-29,548 (SQ, 1 μM), or I-BOP (1 μM) + mI-PTA-PO (5 μM).

CHAPTER 6
CONCLUDING DISCUSSION

The studies in this dissertation were initiated because TxA_2 has been implicated in renal physiology and pathophysiology in previous investigations. Angiotensin II releases TxA_2 which mediates much of the pressor and renal hemodynamic responses of angiotensin II (Wilcox and Welch, 1990a and 1990b). TxA_2 is implicated in tubuloglomerular feedback, i.e. afferent arteriolar vasoconstriction in response to increased delivery to and absorption of NaCl by the macula densa of the distal tubule (Welch and Wilcox, 1988a). Afferent arteriolar vasoconstriction in tubuloglomerular feedback may be potentiated by release of TxA_2 which predominately affects this preglomerular vasculature. Both TxA_2 and angiotensin II promote tubuloglomerular feedback but by independent mechanisms (Welch and Wilcox, 1990). The animal model of human renovascular hypertension with unilateral renal artery stenosis, the 2 kidney, 1 clip rat (2K,1C), is associated with increased renal TxB_2 excretion and response to drugs which reduce TxA_2 generation or antagonize TxA_2/PGH_2 receptors (Himmelstein and Klotman, 1989, Lin et al., 1991). This final chapter contains a general discussion of the importance of the new findings from the individual studies of

the role of TxA_2 in vascular and renal tissues as they relate to one another.

TxA_2 Mimetics have Both TxA_2/PGH_2 Receptor-mediated and Receptor-independent Actions

Thromboxane has receptor-mediated actions in platelets and vascular tissue (Olgetree et al., 1985; Mais et al., 1985) but also receptor-independent actions as a calcium ionophore (Owen and Le Breton, 1981). In isolated aortic rings, the TxA_2 mimetic, U-46,619, stimulated a dose-dependent contraction of aortic rings which was fully antagonized *ex vivo* by the TxA_2/PGH_2 receptor antagonist SQ-29,548. This finding demonstrates that vasoconstiction in response to a TxA_2 mimetic in a major distributive blood vessel is caused by a receptor-mediated mechanism.

Loutenhizer et al. (1986) have demonstrated that a TxA_2 mimetic acts predominately on the renal afferent resistance vessels to decrease the GFR and the RBF by a mechanism that entails voltage-activated calcium channels. In contrast, a TxA_2 mimetic does not change glomerular capillary ultrafiltration coefficient in more sophisticated micropuncture studies (Baylis, 1987). Infusion of U-46,619 in the whole animal in our studies caused a decrease in both GFR and RBF but had no effect on femoral blood flow. These effects on GFR and RBF were fully ablated by SQ-29,548. The dose-response curves showed that U-46,619 was much more potent in increasing RVR than MAP or FVR. Therefore, these

renal hemodynamic responses indicated that a TxA_2 mimetic altered renal hemodynamic functions by a receptor-mediated process in preglomerular blood vessels of the kidney and that renal resistance vessels were preferentially affected.

An enhancement of the renal vasoconstrictive response to U-46,619 is seen in Goldblatt hypertensive dogs in the first two weeks (Zimmerman, 1987). Lin et al. (1991) demonstrate a lowering of blood pressure in renin-dependent hypertension with the TxA_2/PGH_2 receptor antagonist SQ-29,548. In our study of the 2K,1C model of renovascular hypertension, SQ-29,548 reduced hypertension in the early phase and normalized BP in the late phase. These results suggest that the hypertension of 2K,1C rats is partly mediated by TxA_2/PGH_2 receptor activation.

Wilkes et al. (1989) have identified TxA_2 receptor binding in glomeruli and have shown that U-46,619 causes vasoconstriction. Our results confirm these findings. However, whole kidney hemodynamic responses are far removed from receptor binding and may be modified by compensatory neurohumoral processes that do not permit definitive identification of a functional response to TxA_2/PGH_2 receptor binding. In our study I-BOP was used to demonstrate not only a specific binding site in glomerular membranes but also phosphoinositide hydrolysis in isolated glomeruli. These results indicated that a functional TxA_2/PGH_2 receptor is present in the glomerulus, although a relevant physiological response to the TxA_2 mimetic has not yet been identified

there. The large difference in the displacement of [^{125}I]-BOP by stereoisomers illustrates the extremely stringent selectivity of the receptor binding; this was confirmed in parallel studies which showed stereoselective inhibition of I-BOP stimulated phosphoinositide hydrolysis in whole glomeruli. However, in the glomerular mesangial cell, phosphoinositide hydrolysis was stimulated at a much higher EC_{50} than in the glomerulus, even though it was dose-dependent. Since this release of inositol phosphates could not be prevented by TxA_2/PGH_2 receptor antagonists, it must have been stimulated by an as yet unidentified receptor or by a receptor-independent mechanism. Possibly, the TxA_2 mimetic was acting as a calcium ionophore which led to phosphoinositide hydrolysis as an amplification response to the increase in $[Ca^{2+}]_i$ (Eberhard et al., 1991). In preliminary studies glomerular mesangial cells possessed very low specific binding of [^{125}I]-BOP which prevented definitive characterization of the TxA_2/PGH_2 receptor in this preparation but does not obviate the possible presence of a population of TxA_2/PGH_2 receptors in this tissue. Glomerular epithelial cells demonstrated no specific binding sites. Therefore, glomerular endothelial cells or vascular smooth muscle cells which may be extensions of the afferent arteriolar musculature within the core of the glomerulus may contain the TxA_2/PGH_2 receptors that our earlier studies had clearly identified in the glomerulus. Future studies will be directed toward characterizing the TxA_2/PGH_2 receptor in

glomerular endothelial cells and the mechanism of calcium activation in glomerular cells. Autoradiography may be useful to localize the TxA_2/PGH_2 receptor in glomerular, tubular, and vascular structures in the kidney.

Distinct Subtype of TxA_2/PGH_2 Receptors

It has been suggested that there are different subtypes of the TxA_2/PGH_2 receptor in platelets and vascular tissue based on different by rank order of potencies of antagonists in displacing binding ligands from these two tissues (Mais et al., 1985a; Masuda et al., 1991). Infusion of U-46,619 showed a selectivity of action on renal resistance vessels compared to femoral vessels. This result suggests either a differential sensitivity to the TxA_2 mimetic or different subtypes of the TxA_2/PGH_2 receptor in the renal vasculature. Our binding studies showed no correlation between the rank order of potencies of platelet and glomerular membranes for displacement of $[^{125}I]$-BOP by a homologous series of six 13-azapinane TxA_2 antagonists. This finding suggests that the TxA_2/PGH_2 receptor in the glomerulus is different than the receptor in the platelet. However, cDNA clones from human megakaryocytic leukemia cells and placenta, representing the platelet and vascular TxA_2/PGH_2 receptor, respectively, have been isolated and have not demonstrated different subtypes of TxA_2/PGH_2 receptor (Hirata et al., 1991). More studies using molecular biological techniques in other tissues such as

aortic smooth muscle and the glomerulus may show that TxA_2/PGH_2 receptor subtypes exist. Alternatively, our finding of a different pattern of displacement by the 13-azapinane series may be misleading and have a technical explanation, such as differences in the milieu of the receptor in glomerular membranes compared to intact platelets.

TxA_2 Mimetics have Both Direct Vasoconstrictor Actions and an Indirect Action due to Release of Endogenous TxA_2 from Vascular Tissues

The direct actions of a TxA_2 mimetic on vascular tissue are analogous to the direct binding of phenylephrine to the α-adrenergic receptor and subsequent vasoconstriction. The indirect actions of a TxA_2 mimetic are comparable to tyramine-stimulated release of norepinephrine from nerve terminals with subsequent binding to the α-adrenergic receptor and stimulation of vasoconstriction. Platelets release TxA_2 during the secondary or release phase of aggregation has analogy with tyramine, since it is initiated by a receptor-mediated processes but is potentiated by further release of TxA_2 (Moncada and Vane, 1979).

A TxA_2 mimetic releases the metabolites of PGI_2 and TxA_2 from vascular tissue (Mehta et al., 1984). However, a role for this released TxA_2 had not been established. In our isolated aortic ring study, U-46,619 increased release of TxB_2. Moreover, inhibition of TxA_2 release from the rings by a TxA_2 synthetase inhibitor shifted the dose-response curve

for contraction with U-46,619 to the right. This result suggests that the endogenously released TxA_2 increases the sensitivity of the vascular tissue to the TxA_2 mimetic. Infusion of U-46,619 increased the excretion of filtered PGs and TxA_2. Since a TxA_2 synthetase inhibitor reduced the renal vasoconstrictive response to infused U-46,619, I conclude that both in the isolated aortic ring ex vivo and in the renal circulation in vivo, TxA_2/PGH_2 receptor activation not only elicits a direct contractile response but is enforced by an indirect response mediated by endogenous TxA_2 release.

In the 2K,1C model of renovascular hypertension, an increased excretion of TxB_2 was found in both the early and late phases. Angiotensin-induced TxA_2 release was likely during the early phase when the renin activity levels were elevated and BP was normalized by an angiotensin II synthesis inhibitor. In the late phase, the effect of TxA_2 was dissociated from angiotensin II, since the BP remained responsive to SQ-29,548 but was unaffected by any angiotensin II synthesis inhibitor. Thus, renal release of TxA_2 may play a significant role in initiating and maintaining the hypertension in this model.

Activation of phosphoinositide hydrolysis in whole glomeruli by I-BOP may stimulate PLC to release arachidonic acid to form TxA_2. In contrast, I-BOP stimulation of glomerular mesangial cells may activate phosphoinositide hydrolysis by receptor-independent mechanisms which stimulate PLA_2 to release arachidonic acid to form TxA_2. The

endothelium likely possesses the functional TxA_2/PGH_2 receptor in the glomerulus, since we had shown using the isolated aortic ring that U-46,619 released EDRF. Thus, activation of PLC in glomerular endothelial cells may stimulate PLA_2 to release arachidonic acid and ultimately to form endogenous TxA_2. This mechanism may be a relevant physiological response to TxA_2 mimetic binding in glomeruli. Activation of PLC and PLA_2 also may be important in understanding the release of TxA_2 in renovascular hypertension.

The Actions of a TxA_2 Mimetic are Modulated by Release of EDRF, Arachidonic Acid Metabolites, and Catecholamines

The contractile response of aortic rings to U-46,619 was shifted to the left when the endothelium was denuded. This result implies that the sensitivity of the rings to the TxA_2 mimetic was modulated by an endothelium-derived relaxing factor. Since oxyhemoglobin, a scavenger of NO, caused a shift to the left of the dose-response curve when the endothelium was intact, the predominant EDRF was presumably NO rather than PGI_2. The right shift of the contractile response after pretreatment with a TxA_2 synthetase inhibitor indicated that endogenously released TxA_2 increased the sensitivity of the rings to the TxA_2 mimetic. Since this shift occurred whether the endothelium was intact or removed, the TxA_2 must have derived from the vascular smooth muscle. Therefore, both the endothelium and the smooth muscle cells

of large blood vessels activated by a TxA_2 mimetic elaborate modulators which decrease and increase the vascular contractility.

The decreases in GFR and RPF seen with infusion of U-46,619 were markedly reduced by a thromboxane synthetase inhibitor and antagonists of α-adrenergic and leukotriene D_4/E_4 receptors. This findings suggests that not only was endogenous renal TxA_2 released but also that catecholamines were released from renal nerves and that leukotrienes were synthesized in the kidney. Trachte and Stein (1988) has demonstrated a peripheral adrenergic effect of U-46,619 by enhanced norepinephrine release from neurons innervating the smooth muscle of vas deferens, while Sirén et al. (1985) have demonstrated a central cardiovascular effect of U-46,619 by intracerebroventricular injections which increased systemic blood pressure. While the kidney cannot manufacture leukotrienes from arachidonic acid, formation of HETEs (hydroxyeicosatetraenoic acids) by macrophages resident in the kidney may allow synthesis of leukotrienes from this substrate, since renal cells possess all the remaining necessary enzymes (Ardaillou et al., 1986). These results suggest that there is a complex interaction of neurotransmitters which modulate renal hemodynamic functions.

TxA_2 can be Critical in Chronic Blood Pressure Regulation

While plasma renin activity and urinary TxB_2 excretion were elevated 2-fold in both the early and late models of renovascular hypertension, there was a further increase in mean arterial blood pressure in the late phase as compared to the early phase. This finding may indicate a change in the sensitivity to either angiotensin II, TxA_2, or both in systemic or renal vascular tissues. Zimmerman (1987) has shown increased vascular reactivity to a TxA_2 mimetic in the early phase, while the reactivity to angiotensin II does not change. However, the vascular reactivity to a TxA_2 mimetic was not studied beyond 2 weeks, whereas angiotensin was evaluated at 5 weeks. Therefore, the status of vascular reactivity to TxA_2 in the late phase needs further investigation. Okamura et al. (1986) demonstrated that vascular renin activity is increased in the late phase of renovascular hypertension when the plasma renin activity is normal. Thus, there may be different role of circulating renin and local renin produced in blood vessels in the late phase, whereas the sensitivity to TxA_2 in the late phase has not been established.

The renin level in the late phase was not normalized as in some previous studies in the dog (Watkins et al., 1976). Morton and Wallace (1983) in rats and Robertson and Tillman (1987) in humans report elevated renin levels in the late

phase. Nevertheless, the acute administration of an angiotensin converting enzyme inhibitor in our study did not normalize blood pressure in the late phase as it did in the early phase. This confirms previous reports of a lack of effect of angiotensin converting enzyme inhibitors on this phase of 2K,1C hypertension in the rat (Riegger et al.,1977). In contrast, a TxA_2/PGH_2 receptor antagonist normalized the blood pressure within 90 min in our studies.

If a dissociation of circulating and vascular renin activities exists in the late phase (Dzau, 1986), then increased renal vascular renin-angiotensin levels may down-regulate the angiotensin II receptor and thereby reduce the responsiveness to angiotensin II. Another alternative is that while the AT-1 subtype of the angiotensin II receptor may be unresponsive in the late phase, a role for the AT-2 receptor subtype may exist. Further studies of angiotensin receptor subypes and responses may clarify the reason for loss of angiotensin II responsiveness and ascendency of TxA_2 in chronic renovascular hypertenion. However, my studies have shown clearly a surprisingly important role for TxA_2/PGH_2 receptor activation in the maintenance of hypertension throughout the evolution in the 2K,1C rat model.

CHAPTER 7
SUMMARY OF CONCLUSIONS

We conclude from our experiments.

A. The contractile response of aortic rings to a TxA_2 mimetic

1) entails binding to a specific, high-affinity receptor;

2) is offset by release of EDRF from the endothelium;

3) stimulates conversion of arachidonic acid to PGs and TxA_2; and

4) is enhanced by endogenous release of TxA_2.

B. Infusion of a TxA_2 mimetic in the whole animal preparation

1) causes dose-dependent reductions in GFR and RBF which entail activation of TxA_2/PGH_2 receptors;

2) involves vasoconstriction of the renal afferent arterioles;

3) causes a selective increase in renal vascular resistance compared to femoral vessels;

4) stimulates an increase in renal PGs and TxA_2 generation; and

5) induces renal vasoconstriction and a fall in GFR which are mediated in part by release of endogenous TxA_2 and catecholamines and activation of leukotriene D_4/E_4 receptors.

C. In the rat model of 2K,1C renovascular hypertension,

1) plasma renin activity and renal TxA_2 generation are increased in both the early and the late phases;

2) hypertension is entirely dependent on angiotensin converting enzyme activity in the early phase, but becomes resistant after 1-2 months; and

3) PG and TxA_2 production contribute to the maintenance of the hypertension throughout both phases.

D. Specific TxA_2/PGH_2 receptors and function are present in rat glomeruli, as evidenced by

1) binding of a TxA_2 mimetic to glomerular membranes;

2) displacement by a series of TxA_2/PGH_2 agonists and antagonists;

3) generation of a second messenger via phosphoinositide hydrolysis;

4) a close correlation between receptor binding and activation of second messenger responses; and

5) a different subset of the TxA_2/PGH_2 receptor in glomerular membranes versus platelets.

E. Isolated glomerular mesangial and/or epithelial cells

1) respond to a TxA_2 mimetic with an increase in phosphoinositide hydrolysis via a TxA_2/PGH_2 receptor-independent mechanism and

2) do not possess significant specific TxA_2/PGH_2 binding sites.

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BIOGRAPHICAL SKETCH

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